THE PHOTO-OXIDATION OF CHLOROPHYLL

AND RELATED COMPOUNDS

A Thesis for the degree of Doctor of Philosophy

presented by

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INTRODUCTION

The changes which take place when molecular oxygen reacts with an organic compound depend largely on the nature of the compound and the conditions under which reaction occurs. It is thus difficult to give a general mechanism which will cover all such oxidations. In its simplest form this reaction, for which the term 'autoxidation' is used, may be regarded as the addition of molecular oxygen to the compound in question with the formation of a highly reactive peroxide.[1]

The field of autoxidation which has been most widely investigated is that of the saturated and olefinic hydrocarbons, since many of the substances concerned - for example the drying oils and rubbers - are of great practical importance. The complex sequence of reactions involved in these oxidations makes the complete elucidation of the changes taking place difficult. In most cases, however, it is believed that a chain mechanism is involved, and that reaction is initiated by the production of free radicals from the oxidisable molecule either thermally, photochemically or catalytically.[2] Such oxidations are normally irreversible, the primary product being a hydroperoxide which may decompose in turn to give simpler compounds. Other substances such as aldehydes and ethers undergo similarly irreversible reactions with molecular oxygen.

A number of autoxidations are known in which the compounds formed show considerable dissociation at lower pressures or higher temperatures than those under which they were formed.[3] This property of reversible combination with oxygen was first recognised in the complex molecules hemoglobin and hemocyanin, both of which

contain a co-ordinately bound metal atom. The exact nature of the union formed with this type of compound, and the part played by the metal, have not been established.

Similar reversible oxidations have been observed with other compounds which are free from metals. These all contain the basic resonating system of the anthracene nucleus. Addition of oxygen has been shown to take place across a ring in a 1:4-position, giving a transannular peroxide, the dissociability of which is influenced by the substituents in the meso positions. Most of the investigations of this group have been carried out by Dufraisse and collaborators, first on rubrene and then on analogous hydrocarbons.[4]

From a consideration of the structure of chlorophyll[5] it seems possible that it belongs to the group of reversibly oxidisable substances. The oxidation reactions of this compound have been studied primarily from the point of view of their possible importance in photosynthesis, and in this connection a definite proof of reversibility would be of great interest.

The most readily observable reaction of chlorophyll which has been attributed to autoxidation, is the irreversible bleaching which occurs on exposure to light and air. This has been shown to involve an absorption of oxygen. Quantitative results on the subject,[6] however, indicate that the solvent is the final acceptor of part, if not all, of the oxygen taken up. The nature of the process is consequently unknown and is probably complex.[7]

Absorption of oxygen has been observed also in the transformation of chlorophyll known as allomerisation.[8] This change takes place in the dark when alcoholic solutions of the pigment

are exposed to air. The resulting solution is unchanged in colour but it no longer gives the Molisch 'phase reaction'[9], the transient appearance of a brown colour shown by intact chlorophyll on the addition of alcoholic alkali, in the cold and in the presence of air. No allomerisation is found to take place in the absence of alcohol, and this has been explained by Rabinowitch by the assumption that the oxygen taken up initially by the chlorophyll is then transferred to the solvent. The reaction is generally considered to be irreversible, though Stoll and Wiedemann[10] claimed that in the b series at least reversion can be brought about by mild reductants.

Other reactions of chlorophyll which may be mentioned here are the bleaching which occurs on the addition of ferric salts to methanol solutions of the pigment[11], the colour being restored by the addition of a reducing agent, and the reversible photobleaching[12] which is observed only in the absence of oxygen. The first of these changes has been attributed to a reversible oxidation of the chlorophyll by the ferric ions, while the photobleaching is considered to take place by a series of processes which may possibly involve the solvent.

The main difficulty evident in the investigation of these reactions in solution is that of estimating the exact part played by the solvent. A general simplification of this problem might be expected from a study of the solid phase, and in recent years a number of investigations[13] have been carried out in this connection. In these investigations chlorophyll was examined when deposited in the form of thin films on various solid supports, direct measurements being made of the pressure changes

which occurred on illumination of these in oxygen. Previous to this, work on solid films of chlorophyll has been concerned mainly with attempts to detect in the reaction products those organic compounds usually assumed to be involved in photosynthesis.[14]

Since the present work deals with similar solid pigment systems, the general results obtained in these investigations will be given in some detail.

Photo-oxidation was found to proceed at a measurable rate when chlorophyll was adsorbed on thallous bromide crystals; the pressure decrease shown, using a rate extrapolation method, corresponded to a gas pressure decrease to pigment molecular ratio of unity. Reduction in pressure after illumination resulted in a relatively increased rate of pressure change on re-illumination at the same pressure. In this reaction the sole function of the thallous bromide was considered to be that of a true sensitiser.[15]

On the assumption that the pressure decrease observed under such conditions was due entirely to uptake of oxygen, these results were interpreted as corresponding to a partially reversible oxidation of chlorophyll in which one molecule of oxygen was taken up per molecule of pigment. No chain mechanism would appear to be involved because of the relative immobility of the adsorbed molecules. The scheme of reactions proposed to represent the oxidation was as follows —

$$\text{Chl} + h\nu \longrightarrow \text{Chl}^* \qquad (1)$$

$$\text{Chl}^* \longrightarrow \text{Chl} \qquad (2)$$

$$Chl^* \longrightarrow t\ Chl \qquad (3)$$

$$t\ Chl \longrightarrow Chl \qquad (4)$$

$$t\ Chl + O_2 \longrightarrow t\ Chl.O_2 \qquad (5)$$

$$t\ Chl.O_2 \longrightarrow t\ Chl + O_2 \qquad (6)$$

$$t\ Chl.O_2 \longrightarrow Chl.O_2 \qquad (7)$$

where Chl = chlorophyll molecule, Chl^* = electronically excited chlorophyll molecule, t Chl = long-lived excited chlorophyll (tautomer), t $Chl.O_2$ = unstable (reversible) oxide of chlorophyll, and $Chl.O_2$ = stable (irreversible) oxide of chlorophyll.

The kinetic expression derived for the overall reaction rate from this mechanism is of the form

$$-\frac{d[O_2]}{dt} = \frac{a\ I_{abs}\left\{[O_2] - [O_2]_{eq}\right\}}{b + [O_2]}$$

where a and b involve only constants, I_{abs} represents the intensity of the absorbed light, and $[O_2]_{eq}$ is that equilibrium pressure of oxygen at which the rate of formation of reversible oxide is equal to the rate of its decomposition.

From this expression it follows that, apart from the conversion of unstable to stable oxide, the rate of oxygen uptake should become zero at a finite pressure of oxygen. This pressure will equal the dissociation pressure of the reversible oxide. Indications of a pressure limit of oxygen below which no oxidation apparently occurred have already been given by Lonie.[16]

While this conception of reversibility does not seem improbable from a consideration of the chemistry of chlorophyll, its structure and similarities to other reversibly autoxidisable substances, and is not in disagreement with Kautsky's claim that[17]

the kinetics of chlorophyll fluorescence indicate the production of first an unstable oxygen complex and second a non-dissociable peroxide, yet the results on which it is based do not preclude another interpretation which does not involve reversibility. The unit molecular ratio will in general be satisfied by any scheme of reactions in which $(n+1)$ molecules of oxygen are consumed and n molecules of gaseous non-adsorbed product are formed. Thus it is possible that the pressure changes observed refer not simply to the absorption of oxygen, but to an absorption accompanied by the liberation of another resultant gas. The 'reversal' under such conditions must then be due to the removal of some retarding product or reaction, in which an increase in pressure must be concerned. No evidence of any such complicating circumstances was obtained however in the previous work.

A similar investigation[13] of the yellow pigment carotene when deposited on thallous bromide, showed a pressure decrease on illumination in oxygen which was equivalent to a unit molecular ratio. The oxidation was found to be 'reversible' by evacuation, but to a much smaller extent than that of chlorophyll. These results agree with Baur's finding[18] that carotene peroxide is formed only in light with a partially reversible formation, but are, as in the case of chlorophyll, capable at the same time of a different interpretation.

Chlorophyll and carotene are both complex molecules in which there is more than one grouping susceptible to oxidation. Thus in chlorophyll the vinyl group at C_2 (Formula I)[19], the hydrogen atoms at C_7 and C_8, and the 'lone' hydrogen atom at C_{10}, as well as the aldehyde group of chlorophyll b, are all potential centres

of oxidation, while the ready removal of the central magnesium
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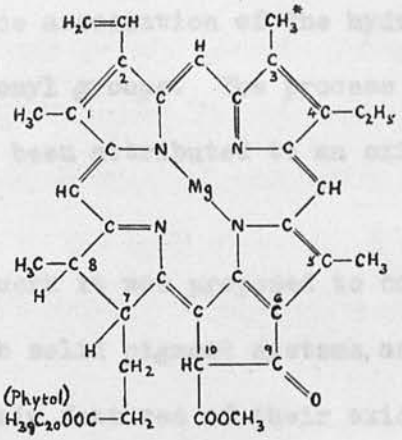
atom by dilute acid solutions presents two further oxidisable positions.



Formula I. Chlorophyll a

(* position of aldehyde group of chlorophyll b)

Carotene also contains several centres of unsaturation, and it has been reported that on exposure to air for several weeks, each molecule takes up about eleven atoms of oxygen before the carbon chain begins to crack and volatile low molecular weight compounds are liberated.

The chemical nature of the reversible and irreversible oxides of the above reaction scheme for chlorophyll photo-oxidation is not
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known, but it has been postulated that attack by the molecule of oxygen takes place at the C_{10} atom, a position known to be particularly reactive. The sequence of reactions on this basis has been considered as consisting of an initial enolisation of the carbonyl group on C_9 by the labile hydrogen atom of C_{10}, induced by light, followed by the addition of oxygen to the double bond

thus formed. The resulting oxide may be the reversible form, production of the final stable form being brought about by the return of the hydrogen atom from its enolised position giving a hydroperoxide. Such a compound would be very stable by reason of the possible resonance association of the hydrogen atom with either of the adjacent carbonyl groups. The process of allomerisation of chlorophyll also has been attributed to an oxidation of the C_{10} position.

In the present work it was proposed to continue the investigation of such solid pigment systems, and to deal in particular with certain features of their oxidation. By a study of the reaction at low oxygen pressures, it was hoped to obtain further information about the changes taking place on illumination, to measure the dissociation pressure of any reversible oxide formed, and to find the effect on that pressure of varying temperature and other conditions. The general reversibility of the oxidation was to be investigated further, and possible alternative interpretations of previous results considered by an examination of the reaction in the presence of added adsorbents. The effect of carbon dioxide and water vapour on the various aspects of the oxidation was also to be examined from the general viewpoint of photosynthesis.

At the same time it was proposed to develop a chemical method of estimating the extent of oxidation of the chlorophyll, and to relate the results obtained to the corresponding experimentally observed pressure changes. Since the final product was considered to be a hydroperoxide, it was possible that the methods used in estimating peroxidic oxygen in oxidised

hydrocarbons might be applied in this case. Several of these methods are known,[22] but the one which seemed most suitable for the detection of the small amounts of peroxide concerned was the colorimetric method of Young, Vogt and Nieuwland.[23] This has been shown to be satisfactory in the presence of unsaturation, and has been applied with modifications to the determination of peroxidic oxygen in large molecules such as rubber.[24] A method of this kind, if proved applicable in the case of oxidised chlorophyll, would be of value in checking conclusions based on total pressure measurements alone.

APPARATUS AND EXPERIMENTAL METHODS

The majority of the experiments described in this work involved the measurement of small pressure changes, and the apparatus used for this purpose is represented diagrammatically in Figure 1.

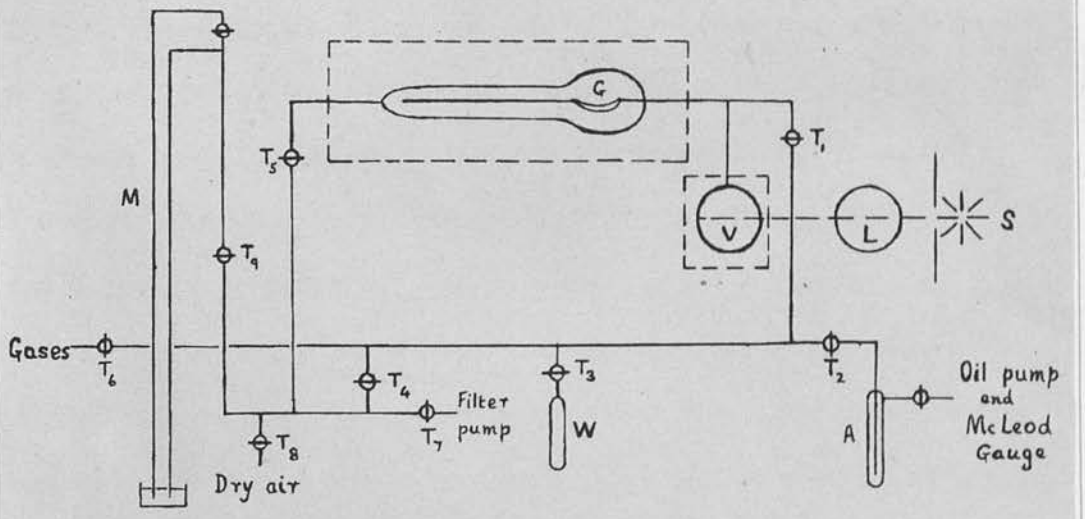


Figure 1.

The reaction vessel V was connected by a ground glass joint and capillary tubing to the Bourdon glass sickle gauge G. This was used to record the small changes in pressure which occurred during reactions, while the absolute pressure of gas within the apparatus was measured on the mercury manometer M. The position of the gauge pointer was read by means of a telescope with scale focussed near its tip, and its movement across that scale calibrated in terms of millimetres of mercury.

The apparatus was constructed entirely of soda glass. Taps and ground joints were lubricated with Apiezon grease L, except where temperatures higher than 25°C were employed. Under such

conditions a Dow Corning Silicone grease was used.

Evacuation of the whole system to a pressure of less than 0·001 mm. mercury could be achieved by a Speedivac pump, the residual pressure being measured on a McLeod gauge attached to the apparatus. During evacuation trap A was generally surrounded by liquid oxygen to prevent any pump oil or mercury vapour diffusing back.

Temperature Control. In the use of the Bourdon gauge at total pressures of 100 mm. it was essential that both it and the reaction vessel be kept at a constant temperature. This was achieved by a water-circulating divided flow arrangement, when the temperature required was the same for both. Water from a thermostat bath was pumped up to a constant head, from which it flowed down in two streams, the one round the gauge jacket and the other round the reaction vessel, and thence back to the thermostat. Such a system of divided water streams tends to cancel out any pressure change effects, as shown by the gauge pointer, due to fluctuation in the thermostat water temperature. A 250 watt carbon filament bulb in conjunction with a chloroform-mercury control and Sunvic control, maintained the temperature of the bath at 25°C. The rates of flow through the gauge and vessel water jackets could be adjusted by screw clips on the rubber tubing from the constant head. During an experiment these were kept at a constant value. The constant head, water jackets, and all connecting tubing, were insulated with asbestos string and felt. Under these conditions, with an adequate flow, the temperature was maintained constant to within ± 0·01°C.

For the investigation of reactions at higher temperatures a

modification of this arrangement was used. While the temperature of the gauge jacket was maintained as before by water circulating from the constant head, that of the reaction vessel was controlled by a separate thermostat bath in which it was placed directly. This bath consisted of an asbestos insulated glass vessel heated externally by a wire mat of variable resistance, which was operated by a small chloroform-mercury control and Sunvic control. The water in this was stirred continuously, except for brief intervals during the reading of the gauge pointer position. At the highest temperature used (75°C) the maximum fluctuation observed was ± 0·5°C, due in part to the time lag involved in the method of heating. Since the pressures concerned in the experiments in which this arrangement was used, were generally less than 0·5 mm., the effect of such fluctuations on the gauge pointer position was negligible.

The temperature of the reaction vessel was changed when necessary during an illumination, by quickly siphoning off the water thermostatting at the higher temperature, and running in water at the lower temperature from the constant head. The level in the vessel bath was maintained by a constant overflow siphon arrangement.

Calibration of the Gauge. The sensitivity of the Bourdon glass sickle gauge was determined by noting the number of telescope scale divisions which corresponded to a change in pressure on the mercury manometer. Several travels of the pointer across the telescope scale were required before a measurable change was obtained on the manometer, and these were brought about by suitably altering the balancing pressures of the gauge and its jacket.

The apparatus was filled with dry air to the pressure for which it was to be calibrated, the manometer reading noted, and the gauge taps T_1 and T_5 closed. A suitable pressure of air was then withdrawn from, or added to, the rest of the system. By opening the gauge taps alternately, the pointer was made to move up and down the telescope scale, and the reading noted at the end of each travel. When the pointer was brought back to its zero position, with both taps open, the final pressure was read on the manometer. The difference in pressure, as given by the manometer, when divided by the total number of scale divisions travelled by the pointer in one direction, either up or down the scale, gave the sensitivity of the gauge in terms of mm. per scale division. During the determination the temperatures of both gauge and vessel were kept constant.

Since telescope scale divisions were read to one tenth of a division, the smallest change in pressure observable with the most sensitive gauge used was 0·0020 mm.

The results of the calibration of one gauge at the temperatures and pressures at which it was used in this work, are given below. Sensitivities quoted in each case are the average of a series of determinations.

Average volume of vessel used (ml.)	Temperature of vessel (°C)	Average absolute pressure (mm.)	Gauge sensitivity (mm./scale div.)
75	25	100	0·0529
75	25	20	0·0533
25	25	20	0·0530
25	75	100	0·0531

Average sensitivity = 0·053 mm./scale division

The sensitivity is seen to be independent of the temperature, the relative volumes of the gauge and its jacket, and of the absolute pressure in the range quoted here. The accuracy of this determination was approximately 3%.

At very low pressures, however, this particular gauge was found to have a different sensitivity. Thus for an average absolute pressure of 0·25 mm., the average sensitivity as found by the McLeod gauge and checked by the manometer, was 0·041 mm. per scale division, both at 25°C and at 75°C. Theoretically the sensitivity of such sickle-type gauges does depend on the absolute pressure.

Reaction Vessels. The vessels used in most experiments were spherical in shape, of soft glass, and approximately 75 ml. in volume. The pressure changes observed in certain reactions using these, were too small for accurate rate determinations and smaller vessels had to be employed. They were approximately one third of the volume of the spherical ones and were almost cylindrical in shape, having one convex face on which films were deposited. While these magnified the observed reaction rates, they also tended to increase any effects due to temperature fluctuations in the thermostatting system. Such effects were unavoidable and resulted from the inequality in volume of the gauge water jacket and the vessel water jacket.

The investigation of reactions in the presence of adsorbents was carried out in a special vessel having a detachable side tube. This tube was joined to the neck of the vessel by a ground glass joint and contained the adsorbent.

Volumes of Vessels

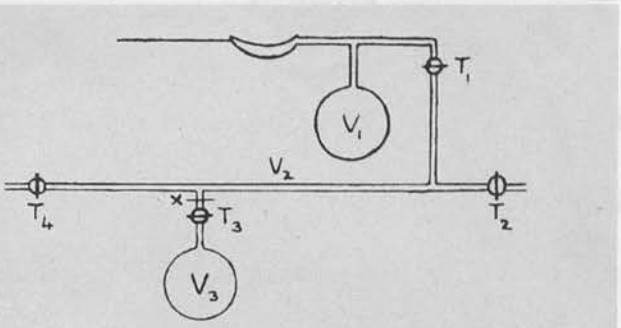


Figure 2.

The volume of the reaction space V_1 (Figure 2), which consists of the vessel, gauge sickle, and capillary connecting tubing to tap T_1, was found by expanding the gas occupying this space at a known pressure and constant temperature, into a subsidiary bulb of known volume. This bulb was attached temporarily to the apparatus, and its volume V_3 determined beforehand, when cut off at the point X, by weighing first empty and then full of distilled water, up to and including the capillary of the tap T_2.

Dry air at room temperature was admitted to the apparatus to a pressure P_1, which was read on the manometer. With taps T_1 and T_4 closed, the connecting tubing of volume V_2 and the subsidiary bulb V_3, were evacuated through T_2. The tap T_3 to this bulb was then closed, and the gas in V_1 expanded into the connecting tubing. The pressure in the gauge jacket was reduced simultaneously to maintain the pointer in its initial position on the scale, and the new pressure P_2 noted. The gas in the volume $(V_1 + V_2)$ was then expanded in the same way into the bulb V_3, through tap T_3, and the final pressure P_3 read.

The actual results obtained in a typical case were as

follows:

Volume of bulb = V_3 = 86·3 ml.

P_1 = 298·3 mm., P_2 = 221·8 mm., P_3 = 122·9 mm.

By Boyle's Law $(V_1 + V_2 + 86·3)\ 122·9 = (V_1 + V_2)\ 221·8$

$$\frac{V_1 + V_2 + 86·3}{V_1 + V_2} = \frac{221·8}{122·9} = 1·805$$

$$\frac{86·3}{V_1 + V_2} = 1·805 - 1 = 0·805$$

$$\therefore\ V_1 + V_2 = \frac{86·3}{0·805} = 107·2$$

Also $(V_1 + V_2)\ 221·8 = (V_1)\ 298·3$

$$\therefore\ V_1 = \frac{107·2 \times 221·8}{298·3} = 79·7 \text{ ml.}$$

And V_2 = 27·5 ml.

Repetition of this determination gave results concordant to within ± 0·2 ml.

Source of Light. Illumination in all experiments was provided by an Osira mercury lamp (125 W. 230 V.). The beam of light from this was focussed by a spherical water lens L (Figure 1) on to the back face of the reaction vessel, the water serving at the same time as a heat filter. After passage through the soft glass of this optical system, the resulting 'white' light contained no light of wavelength less than 3650 Å.

Gases. The oxygen and carbon dioxide used were taken from cylinders and stored in glass bulb reservoirs attached to the apparatus. When required, these were admitted to the system through tap T_6. Before entering its reservoir the cylinder

oxygen was dried by passage over phosphoric oxide, then freed from any condensable impurities by a carbon dioxide/ether or liquid oxygen trap. The cylinder carbon dioxide, after being dried in the same way, was collected directly in a bulb, then frozen out into a trap by liquid oxygen. Uncondensed gases were pumped off and the carbon dioxide then allowed to expand back into the bulb. This process was repeated to remove any impurities trapped in the carbon dioxide on first freezing.

Water vapour was obtained under reduced pressure from the distilled water tube W (Figure 1). It was generally admitted directly to the reaction vessel, tap T_4 being closed, and the pressure balanced by the addition of dry air to the gauge jacket from the calcium chloride tower attached to T_7.

Preparation of Solutions - Chlorophyll and Carotene

Three preparations of chlorophyll were examined in the present work, two from commercial pastes supplied by J.F. Macfarlan & Co., and the third obtained by direct extraction from spinach leaves. The chromatographic procedures adopted in these preparations were designed to separate chlorophyll from carotenoids, no attempt being made to separate the chlorophyll fraction into the chlorophylls a and b.

The crude pastes consisted of mixtures of plant pigments. Solutions were prepared from these by shaking with acetone in the dark for several hours (approx. 1 g. paste in 100 ml. acetone), then filtering off the insoluble residue. The concentration of the resulting stock solution was found, and this when diluted suitably was used in certain experiments where a mixture of chlorophyll and carotenoids was required.

Separation of these pigments was achieved chromatographically using bonemeal as adsorbent. This was chosen partly because of its relative inertness, other common adsorbents such as alumina having been known to cause degradation of chlorophyll,[25] and also because of its efficiency in separating carotenoids from chlorophyll.[26] Before use, the crude steamed bonemeal was repeatedly extracted with hot alcohol, washed with petroleum ether and acetone, dried, and ground until it passed a 100 mesh sieve. The column, of dimensions approximately 8 x 2 cm., was washed with petroleum ether (60-80°) before the addition of the pigment solution. 25 ml. of the stock solution was evaporated to remove acetone, taken up in petroleum ether (60-80°), and added to the column. On developing with the same solvent, the green chlorophyll fraction was found to remain adsorbed near the top of the column, while the yellow carotenoid region moved down. Removal of this fraction was complete after the addition of approximately 300 ml. petroleum ether, the eluate then being colourless. The chlorophyll fraction was then eluted easily by acetone, except for a dark green band which remained at the top of the column. Extensive washing with various solvents failed to remove this, and it was discarded, being considered a degradation product very strongly adsorbed by the bonemeal. Each fraction was then filtered and its concentration determined.

The plant chlorophyll solution was obtained from spinach leaves by a modification of the method described by Zscheile and Comar[27] for the preparation of spectroscopically pure chlorophyll. About one kilogram of fresh clean spinach leaves was extracted with acetone by shaking vigorously for several hours

in dark bottles, sufficient calcium carbonate having first been added to the solvent to neutralise any plant acids liberated. The mixture was filtered by suction, washed with acetone, and the residue re-extracted. This left the cell fragments almost colourless. Altogether approximately 4 litres of acetone was used in the extraction. The extract was then added in one litre portions to a solution containing 1 litre petroleum ether (38-40°C) and 100 ml. acetone in a large separating funnel. After each addition, the mixture was gently rotated and distilled water added until two layers formed. The water-acetone layer was removed, in this way getting rid of most of the water and acetone soluble impurities. The petroleum ether solution was washed several times with water, then three times with 250 ml. portions of 80% methyl alcohol. This served to eliminate most of the xanthophylls. Before use, the methyl alcohol was saturated with petroleum ether, and 0·01 g. oxalic acid added per litre to prevent allomerisation. The solution was then washed repeatedly with distilled water to remove methyl alcohol and any remaining acetone, the washings discarded, and the petroleum ether solution dried over anhydrous sodium sulphate.

Sucrose was used for the purification of this solution of crude chlorophyll, as recommended by Zscheile[27] and Comar. Previous work[28] has shown that the product of such purification does not differ significantly in absorption spectrum from similarly extracted material separated on bonemeal. The bulk of the carotenoids were first removed from the petroleum ether solution by passing it through a tightly packed layer of sucrose on a large Buchner funnel. After repeated washing with the same

solvent, the layer was mechanically removed and extracted with ether. The resulting solution was made up with twice its volume of petroleum ether, and adsorbed on a column of icing sugar, 40 × 5 cm. Before use as an adsorbent the sugar was dried in vacuum at 80-90°C. The column was developed with petroleum ether until separation of the chlorophyll and remaining carotene was achieved. After draining, it was extruded and the chlorophyll fraction containing the a and b components extracted with diethyl ether. The resulting solution of pure chlorophyll was shaken with distilled water to remove sugar, dried over anhydrous sodium sulphate, and finally filtered through sintered glass.

All operations involved in the preparation of these solutions were carried out in very dim yellow light. The solutions finally obtained were stored in the dark and used as soon as possible after preparation.

One of the commercial chlorophyll preparations contained copper as a stabiliser present to the extent of 0·7%. Previous work has shown[29] that this copper content is not affected by the chromatographic separation, and the metal is therefore considered as replacing the central magnesium atom of the chlorophyll molecule[30], approximately one molecule in ten being thus affected. This copper-containing chlorophyll solution differed from the other two preparations in that it showed no fluorescence in ultra violet light and gave a negative Molisch phase reaction[9]. Its absorption spectrum agreed with that found by Rabinowitch and Weiss[11] for pure chlorophyll, and these differences were therefore attributed[31] to the effect of the copper present.

The three chlorophyll preparations will be designated as

copper chlorophyll, copper-free chlorophyll, and spinach chlorophyll.

Pheophytin. Magnesium-free chlorophyll, or pheophytin, was prepared by a small scale adaptation of the method used by Willstätter[32] for a direct preparation from grass. 25 ml. of the bonemeal purified solution of copper-free chlorophyll (0·79 g./litre in acetone) was shaken in the dark for one day with 0·035 g. analar oxalic acid crystals. Extraction of the resulting solution with a chloroform-water mixture was followed by repeated treatment of the extract with water, until the washings gave no reaction on heating with acid permanganate. The chloroform solution was evaporated, dried over phosphoric oxide to constant weight, and then dissolved in a measured volume of acetone. This solution was found to give the phase test and to be fluorescent in ultra violet light, reactions characteristic of both chlorophyll and pheophytin. Its absorption spectrum was compared with that reported by Zscheile[27] and Comar for pheophytin.

Concentration of Solutions. This was found by the removal of the solvent from measured portions of the solution in question, followed by evaporation under vacuum in the dark until constant weight was attained. The process was carried out either in a weighed crystallising dish placed in a vacuum desiccator, or at the oil pump in a weighed bulb attached to the apparatus, using a liquid oxygen trap. Duplicate determinations were made for each solution.

Absorption Spectra. The determination of the absorption spectra of the chlorophyll solutions was used as a means of checking the purity and intactness of the preparations, the results obtained

being compared with those reported for the pure compound.

Measurements were made visually, using the Hilger Constant Deviation Wavelength Spectrometer in conjunction with a Barfit Nutting Photometer, and according to the general method outlined by Twyman and Allsopp.[33] The instrument was calibrated by replacing the pointolite source used in the determinations, by an Osira mercury lamp, and adjusting the pointer in the field of view until it coincided with the band of known wavelength selected by the pointer on the wavelength drum. Solutions diluted to approximately 0·15 g. per litre were used with cells of exactly 1 cm. length. The absorption measurements were made at 100 Å intervals over the range 7000 - 4500 Å, and at 50 Å intervals in the region of the spectral maxima.

Preparation of Films. The pigment to be examined was deposited from solution in the form of a thin film on thallous bromide or some other substrate. The thallous bromide used was prepared by the slow addition of 50 ml. of thallous nitrate solution (5·32 g. in 100 ml.) to 25 ml. of potassium bromide solution (5 g. in 100 ml.), with continuous shaking in a dim yellow light. Precipitation under these conditions occurred in the presence of excess bromine ions. The thallous bromide was washed several times by decantation with distilled water, then dried over phosphoric oxide, powdered and stored in a desiccator. Other substances used were thallous iodide, prepared in a similar way to this, and powdered glass, either Winchester or Jena. The latter was washed thoroughly with acetone and dried before use.

Variation in the amount of thallous bromide taken to prepare the film showed that a maximum initial reaction rate for the

usual conditions was obtained with 0·3 g. The retardation observed with greater amounts of solid, was ascribed to screening or to increased scattering of light by the crystals. 0·3 g. was used therefore in all the experiments, except those carried out in the small reaction vessels. The amount taken in such cases was decreased to correspond with the reduced surface area. With other substrates the weights used were the same.

In preparing the film, the weighed quantity of dry, finely powdered solid was added to the reaction vessel. The required volume of pigment solution was then introduced by means of a calibrated pipette, the narrow stem of which projected down the neck of the vessel and allowed the solution to drop directly on to the substrate. The solvent, normally acetone, was removed by evaporation at room temperature at the filter pump. During the latter stages of the evaporation, the vessel was rotated gently in such a way as to spread out the thallous bromide, giving a smooth film on one side of the vessel. Any chlorophyll inadvertently deposited on the uncovered surface of the vessel during this process, was transferred back to the film by careful washing with a few drops of solvent, followed by evaporation as before. The complete operation was performed in a dim yellow light. When the film was dry, the vessel was attached to the main apparatus, in such a position that the film would be completely illuminated, and evacuated thoroughly by oil pump. After standing in vacuo overnight, it was evacuated further and 'washed out' with oxygen before beginning an experiment.

General Procedure. The experiments to be described in this work can be divided into two sections: (a) those carried out at

low oxygen pressures ($< 0{\cdot}5$ mm.), and (b) those at high oxygen pressures (100 mm.). Since the procedures involved are different, an outline of the method used in each case will be given, before proceeding to the actual results obtained.

(a) Low pressure oxidations. After 'washing out' with oxygen, evacuation of the system was continued until the residual pressure was less than 0·001 mm. Taps T_1 and T_5 (Figure 1) were then closed, and a few millimetres oxygen admitted to the rest of the apparatus. By carefully opening T_1, oxygen was allowed to enter the vessel until the gauge pointer had risen by the required amount. The difference between the initial and final readings on the scale, represented the initial pressure of oxygen in the vessel. When, after a suitable period, it had been ensured that no movement of the pointer was taking place in the dark, the film was illuminated. The decrease in pressure which occurred then was followed by reading the position of the pointer on the scale at intervals. From the graph of pressure against time, the rates of reaction at various pressures were calculated and plotted against those pressures.

(b) Oxidations at high pressures. The process of evacuation and 'washing out' in this case was followed by the introduction of oxygen to the whole system, until the required pressure (usually 100 mm.) was registered on the manometer. Taps T_1, T_4 and T_5 were then closed, and after allowing time for constant temperature to be reached, as shown by the steady position of the gauge pointer, the film was illuminated. From the readings of pressure against time, rates were calculated and plotted in these experiments against the corresponding pressure decreases.

Since the total decrease in pressure in most of these reactions was less than 1 or 2 mm., the oxygen pressure could be taken as constant at 100 mm.

The graphs of rate against pressure and rate against pressure decrease, are referred to as 'rate curves'. These rates represent a decrease in pressure, but for convenience are plotted as a positive quantity, and are expressed as 'scale divisions per 10 (or 100) minutes'.

The position of the gauge pointer on the scale could be altered at any time during an experiment without disturbing the gas in the reaction vessel. Thus by either increasing or decreasing the balancing pressure in the gauge jacket, the pointer was moved down or up the scale, and in this way readings were taken only in that region of the scale in which it was best focussed.

Care was required in the introduction to and removal of gases from the system, since the difference in volume of the gauge and its jacket caused considerable fluctuation of the pointer during such operations. The maximum pressure difference which could safely be allowed between the gauge and its jacket, depended on the sensitivity of the gauge in question, but was of the order of 10 mm.

The addition of other gases during an oxidation was achieved by evacuating the tubing between taps T_2 and T_6, filling it with the required gas to a pressure greater than the original by a few millimetres, and then opening taps T_1 and T_5 and allowing the gas to flow in till the desired pressure was reached.

During the periods between successive illuminations of a

film, when taps T_1 and T_5 were open, the manometer tap T_9 was closed to prevent any possibility of mercury vapour diffusing to the film.

Examination of Reaction Products. The gases present after the illumination of pigment films in oxygen, were frozen out in a number of instances and examined for reaction products such as peroxide and aldehyde. This was done by drawing the gas slowly through a trap containing a little distilled water surrounded by liquid oxygen. After thorough evacuation and 'washing out' of the system with oxygen, the trap was allowed to come to room temperature and then removed from the apparatus. The solution was tested with Schiff's reagent for aldehyde, and with chromic acid/ether or titanic sulphate for peroxide reactions.[34]

The film itself was examined on removal, either by extraction with water and testing the extract in the same way, or by dissolution of the oxidised pigment in acetone and determination of its absorption spectrum.

After certain experiments, the gases present were tested for carbon dioxide by freezing out in a trap containing a solution of baryta. An approximately 0·01 normal solution was used, which had been prepared and stored as described by Cumming and Kay.[35] The procedure adopted in estimating the amount of carbon dioxide was as follows. The air in the glass leads between the main part of the apparatus and the trap, was displaced by carbon dioxide-free air drawn by suction through a soda lime tower. 2 ml. of the baryta solution was pipetted into the trap, taking care to exclude air as far as possible during the addition, the trap surrounded by liquid oxygen and evacuated. The gas to be tested

was then drawn slowly through. After thorough evacuation of the reaction vessel, followed by 'washing out' with oxygen, the trap was closed off, the refrigerant removed, and the solution allowed to come to room temperature. Air free from carbon dioxide was then admitted, and the solution titrated immediately with a 0·01 normal solution of hydrochloric acid, using phenolphthalein as indicator. At the end point, a further 2 ml. baryta solution was added to the trap and the solution retitrated. This served as a correction for any small error caused by the action of a local excess of hydrochloric acid on the precipitate. The difference in the titrations is equivalent to the amount of carbon dioxide present in the sample of gas.

The examination of a number of 'film residues', obtained by extraction with acetone, alcohol or ether of partially oxidised films of chlorophyll on thallous bromide or powdered glass, using ferrous thiocyanate reagent in methanol, showed that some at least of the oxygen taken up on illumination was present as a peroxide. The colorimetric method of estimating such peroxides, developed by Young, Vogt and Nieuwland[23], was therefore applied with some modification in the present work.

The reagent used was prepared by dissolving 5 g. ammonium thiocyanate in approximately 500 ml. of pure methanol, adding a few drops of concentrated sulphuric acid, and making the volume up to 1 litre with methanol. The sulphuric acid apparently acts as a preservative for the solution. Before each determination, sufficient ferrous ammonium sulphate[36] was added to the required volume of this solution to saturate it, and after shaking, the resulting solution of ferrous thiocyanate was decanted and used immediately.

In determining the amount of peroxidic oxygen present after the illumination of a film of chlorophyll in oxygen, it was extracted with pure methanol, the extract filtered, 10 ml. of the reagent added, and the volume of the solution made up to 25 ml. with methanol. After standing for 20 minutes, the colour developed was compared against that in the reagent using the Spekker Photo-electric Absorptiometer (Adam Hilger Ltd., London), with Spectrum Green 604 filters and cells of 1 cm. length. Since the absorption due to the unoxidised chlorophyll, though small, was not negligible, the colour developed in the oxidised solution was compared also against that developed in a solution containing approximately the same amount of unoxidised chlorophyll together with an equal volume of reagent and methanol. This gave two values for the colour produced in the oxidised solution, the one representing the total absorption of the ferric thiocyanate complex and the remaining chlorophyll, and the other an approximate value of the absorption due to the complex alone. The estimation with reagent was made as soon after dissolution of the film in methanol as possible, to prevent any complications arising from an interaction of chlorophyll and the solvent.[9]

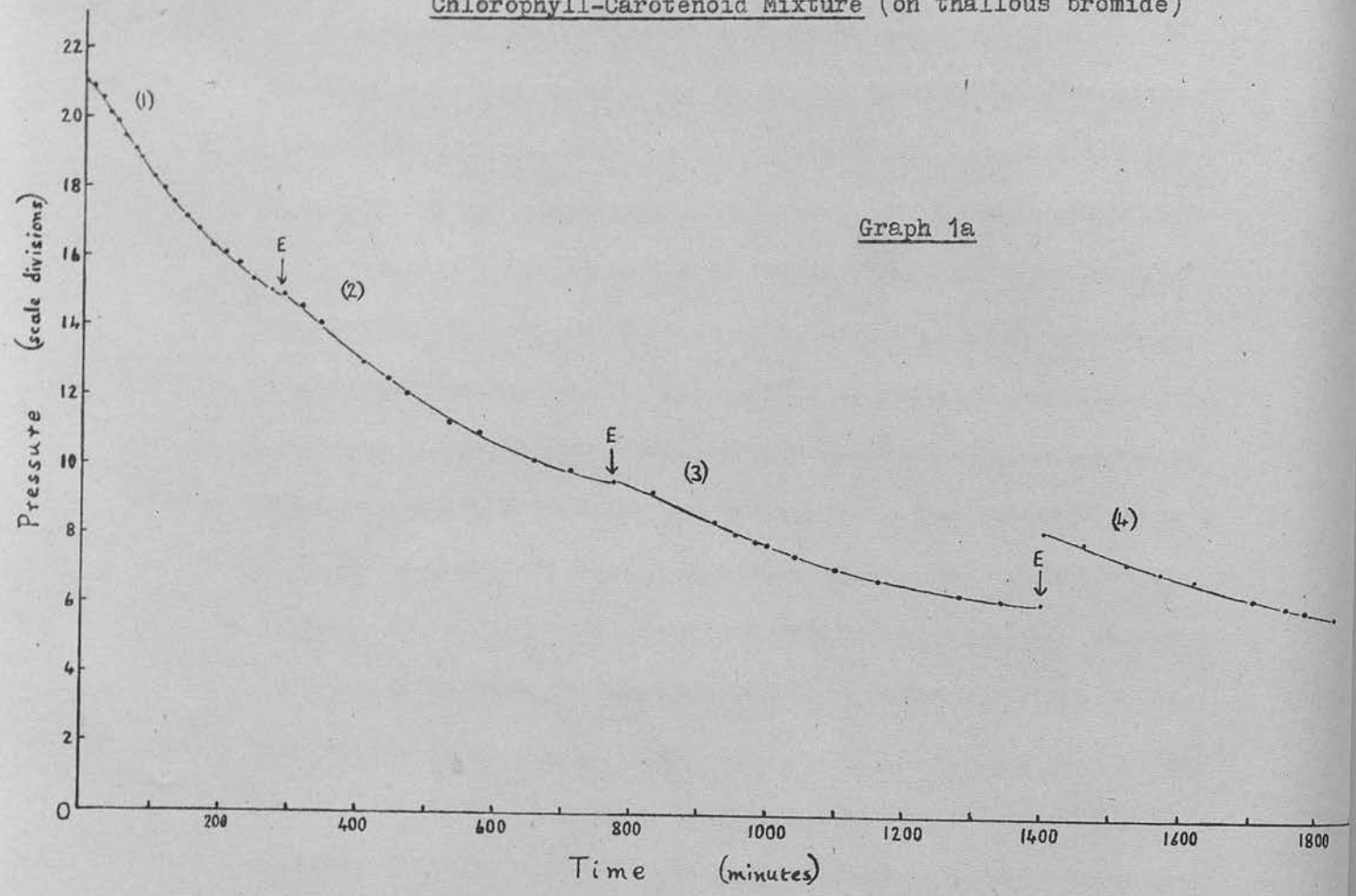
For results of absolute significance, it was necessary to calibrate the instrument by measuring the colour produced by peroxides of known concentration. Two calibration curves were prepared, the one from standard solutions of hydrogen peroxide in methanol, and the other from solutions in the same solvent of decalin hydroperoxide, an organic peroxide known to be reasonably stable.[37] [Thanks are due to Dr. C.F.H. Tipper for supplying the sample of decalin hydroperoxide.]

(1) Hydrogen peroxide calibration curve. An approximately decinormal solution of commercial hydrogen peroxide was prepared, and its concentration found by direct titration with decinormal potassium permanganate, previously standardised by the oxalic acid method.[38] To known volumes (1 ml. or less) of suitable dilutions of this solution, were added 10 ml. ferrous thiocyanate reagent plus methanol to 25 ml. The colour developed in each was compared against that in the reagent after a 20 minute interval, using Spectrum Green 604 filters and cells of 1 cm. length. The curve obtained by plotting the direct drum reading ($\log \frac{I_0}{I}$) against the number of micrograms peroxidic oxygen in 25 ml., was found to be linear up to a concentration of approximately 30 µg. peroxidic oxygen.

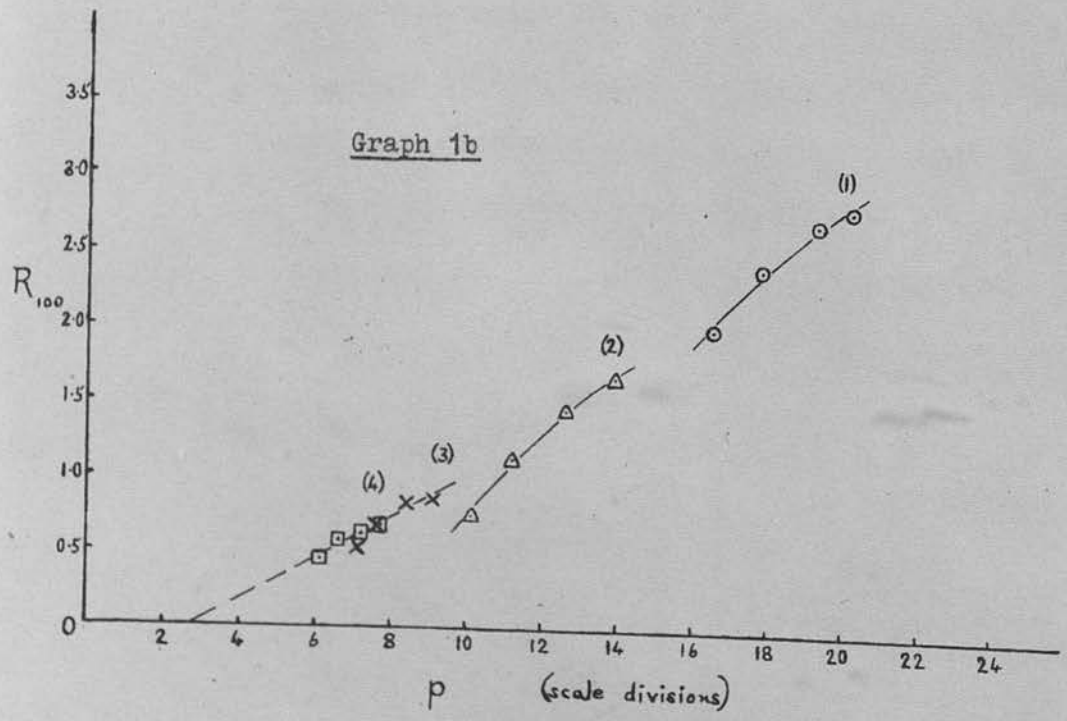
(2) Decalin hydroperoxide calibration curve. Measured volumes of a solution of decalin hydroperoxide in pure methanol (7·87 mg. in 50 ml.), were taken and to each 10 ml. ferrous thiocyanate reagent was added plus methanol to 25 ml. The colour developed was measured as before, after 20 minutes, using Spectrum Green filters. The same range of peroxidic oxygen concentration was covered as with hydrogen peroxide, but the curves obtained were not found to be exactly equivalent at all points. In this case a smaller portion of the curve was linear.

Chlorophyll-Carotenoid Mixture (on thallous bromide)

Graph 1a



Graph 1b



EXPERIMENTAL RESULTS

Photo-oxidations at Low Oxygen Pressures

The kinetic expression (p. 5) derived to represent the rate of photo-oxidation of chlorophyll, includes constants and the variable terms I_{abs} and $\{[O_2] - [O_2]_{eq}\}$, where $[O_2]_{eq}$ is the dissociation pressure of the reversible oxide formed. Preliminary experiments by Lonie[16] on a chlorophyll-carotenoid mixture in the pressure region below 0·5 mm., showed that this critical pressure must be of the order of 0·1 mm. of oxygen. With a view to the further investigation of such a pressure limit, this experiment was repeated and the results are given below.

Chlorophyll-Carotenoid Mixture (on thallous bromide)

Experimental. 3·12 mg. chlorophyll-carotenoid mixture (copper-free sample) were deposited from acetone solution on 0·3 g. of thallous bromide in the spherical reaction vessel. After evacuation of the system, oxygen was added to the vessel to give a rise in scale reading of 21·0 divisions, equivalent to a pressure of 0·42 mm. mercury (gauge sensitivity = 0·020 mm. per scale division). The film was then illuminated by 'white' light at a temperature of 25°C, and observations of the position of the gauge pointer made at intervals.

The plot of pressure against time for this and subsequent illuminations of the same film, was of the form shown in Graph 1a. At the positions marked E on the graph, illumination was interrupted and the system pumped out. After a period in vacuo a fresh small amount of oxygen was added and illumination recommenced. Initial oxygen pressures in the subsequent illuminations were

(2) 14·9 div., (3) 9·5 div., and (4) 8·1 div. The rates of pressure change obtained from these pressure-time curves were as follows -

	p	R_{100}		p	R_{100}		p	R_{100}		p	R_{100}
(1)	20·2	2·80	(2)	13·9	1·67	(3)	9·1	0·85	(4)	7·7	0·67
	19·3	2·70		12·6	1·46		8·4	0·82		7·2	0·62
	17·8	2·40		11·2	1·12		7·6	0·67		6·6	0·58
	16·5	2·00		10·1	0·75		7·1	0·53		6·1	0·46

where p = pressure at any instant expressed in scale divisions and R_{100} = rate of pressure decrease at the same instant expressed in scale divisions per 100 minutes.

These results may best be treated by an extrapolation method in which rates are plotted against the corresponding pressures, as shown in Graph 1b.

In comparing rates with pressures in general, for the illumination of any one sample, it would be expected from previous results at a constant pressure of oxygen (say 100 mm.), that the rate would decrease because of decreasing chlorophyll concentration causing a decreased light absorption, I_{abs}. Where, however, the total change in pressure during any one illumination is small, so that the change in pigment concentration is negligible, the previous kinetic expression (p. 5) indicates that the plot of rate against pressure should be a straight line, provided that the incident light is constant.

This would appear to be the case in runs (3) and (4), where the rates obtained from separate illuminations lie on the same straight line. Extrapolation of this line shows that zero rate of oxidation would be reached at a finite pressure of oxygen,

namely 2·8 div. ≡ 0·056 mm. In view of the 'reversibility' which has been observed with this sample of chlorophyll[13b,c], this extrapolated limit would appear to represent the pressure at which the rate of formation of reversible oxide is equal to the rate of its dissociation.

The non-linearity of the rate curves (1) and (2) may be accounted for by the more appreciable variation in chlorophyll concentration, resulting from the greater pressure changes at the higher oxygen pressures.

Since these results may be complicated by the fact that both chlorophyll and carotene are present, similar examinations of the bonemeal separated fractions were made. Unless otherwise stated, the following experiments were all carried out at 25°C, in the spherical vessels, using 'white' light illumination.

Chlorophyll Fraction (on thallous bromide)

Experimental. 2·65 mg. carotenoid-free chlorophyll (copper-free sample)

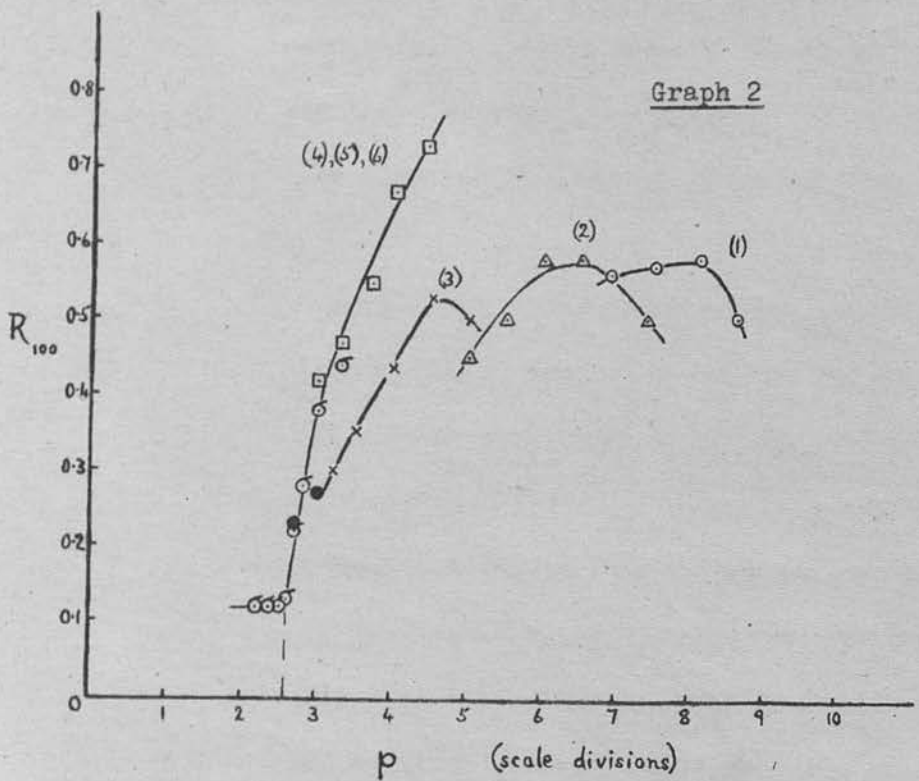
0·3 g. thallous bromide

Gauge sensitivity = 0·020 mm./scale division.

From a series of illuminations of this film, interrupted by periods in vacuo, pressure-time curves were obtained of the same general form as with the chlorophyll-carotenoid mixture. Initial oxygen pressures in these illuminations were (1) 9·0 div., (2) 7·7 div., (3) 5·5 div., (4) 5·3 div., (5) 3·7 div., (6) 3·5 div., and the rates obtained as follows -

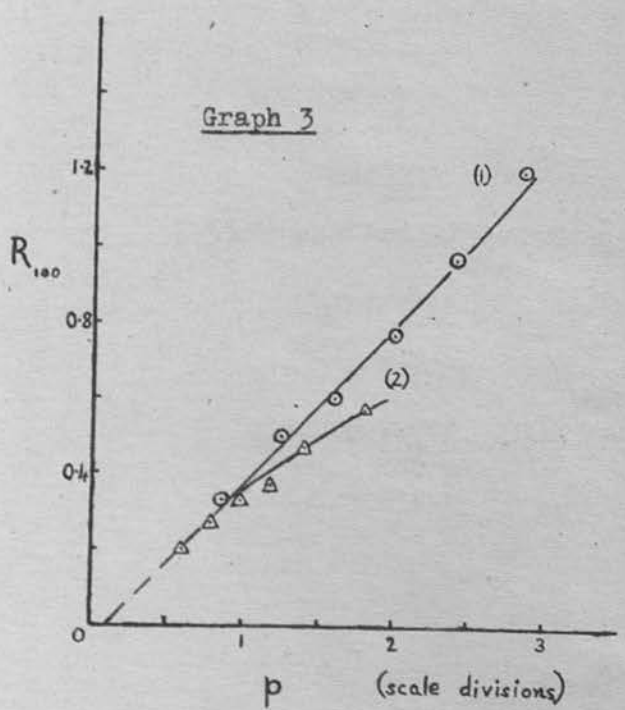
Copper-free Chlorophyll (on thallous bromide)

Graph 2



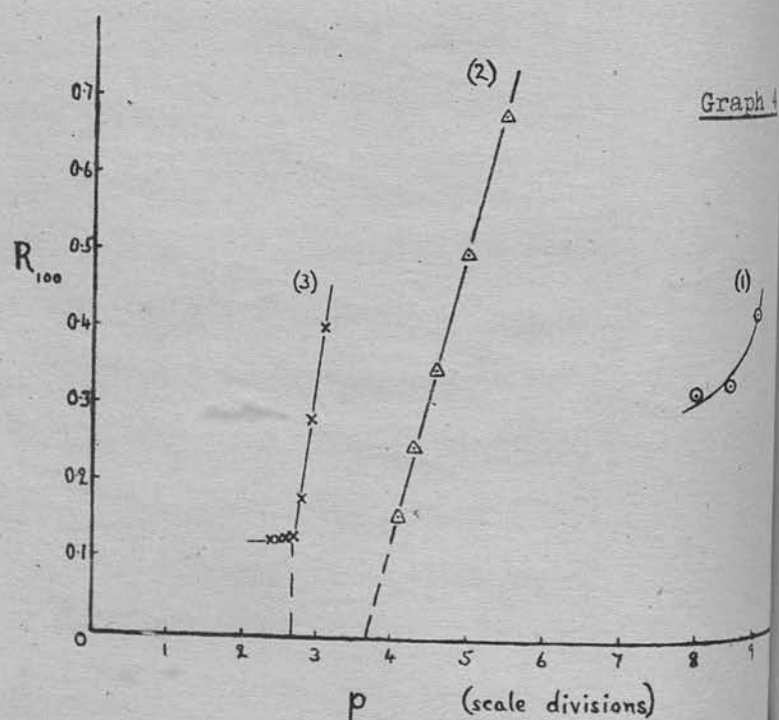
Carotene (on thallous bromide)

Graph 3



Copper-free Chlorophyll - in presence of CO_2
(on thallous bromide)

Graph 4



	p	R_{100}		p	R_{100}		p	R_{100}		p	R_{100}
(1)	8·6	0·50		5·5	0·50	(4)	4·4	0·73		2·8	0·28
	8·1	0·58		5·0	0·45		4·0	0·67		2·7	0·22
	7·5	0·57	(3)	5·0	0·50		3·7	0·55		2·6	0·13
	6·9	0·56		4·5	0·53		3·3	0·47		2·5	0·12
(2)	7·4	0·50		4·0	0·44		3·0	0·42		2·4	0·12
	6·5	0·58		3·5	0·35	(5)	3·3	0·44		2·2	0·12
	6·0	0·58		3·2	0·30		3·0	0·38	(6)	3·0	0·27
										2·7	0·23

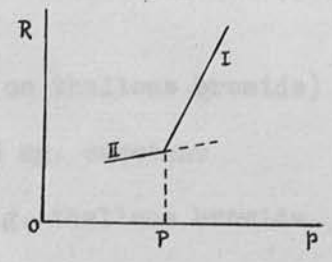
After run (4) a further 7·94 mg. chlorophyll was added to the film, and oxidation recommenced after evacuation. Normally such an increase in concentration would tend to increase the absolute rate of reaction, and in view of the low rates involved here would seem desirable. In practice, however, no marked increase was observed. The absolute rate at the low pressures concerned appears, therefore, to be determined mainly by the oxygen pressure, and not by I_{abs}.

Graph 2 shows the rate curves for this sample of chlorophyll. Pronounced induction or acceleration periods are evident in all the initial curves, and it is only at pressures in the region of 0·06 mm. that near linearity is reached. Rates from different illuminations in this region are found to coincide, and extrapolation of the linear portion of the curve cuts the pressure axis at p = 2·4 div. ≡ 0·048 mm.

The provisional kinetics developed so far (see p. 4) visualises one reversible oxidation product of chlorophyll and one irreversible final stage, the latter being much slower than the first, since evacuation has been found to restore the original condition to a certain extent. The relative rates of the two

reactions will depend on the relative amounts of the reacting species, and will involve the question as to whether the unstable oxide is light sensitive. After the initial stages of the illumination, the curves obtained must represent both processes. Below the dissociation pressure the assumed mechanism indicates that no oxidation should take place, and any further pressure decrease then occurring must correspond to the conversion to the stable oxide.

In the prolonged illumination of run (5), at an initial oxygen pressure of 0·074 mm., it was found that after about 5 hours the rate of pressure decrease was exceptionally slow ($R_{100} = 0{\cdot}12$), and remained, as far as could be judged, the same over the next 6 hours. This slow steady rate may then represent the conversion of chlorophyll to the stable oxide; if illumination could be continued sufficiently long this rate must eventually decrease until zero oxygen pressure is reached. In view of this a correction must be applied to the limit of 0·048 mm. obtained by extrapolation, in order to find the true critical pressure. Thus the complete rate curve may be considered as consisting of two portions -



Near the point of intersection of curves I and II, the rate curve II may be taken as given by its projection as shown, and the true curve I - and hence the true limit - may be obtained by the subtraction of this curve from the combined curve I, because during curve I both processes are in operation. This subtraction

will not be valid over a wide range since the amounts of oxidisable and reversible materials will not be constant. The same result will be obtained by finding the intersection (OP) of the two portions of the curve, in the present instance the intersection of curve I with the 0·12 level. Later work showed that this sample of chlorophyll contained some pheophytin, which may therefore also contribute to the slow residual rate.

From Graph 2 the pressure of 0·052 mm. would then appear to be the best approximation to the equilibrium pressure for the dissociation of the oxide of chlorophyll at 25°C.

Comparison of this result with that for the chlorophyll-carotenoid mixture, shows that the dissociation pressure observed with the latter was due mainly to the chlorophyll present.

Rates in this experiment at pressures considerably above the critical limit, are much lower than might be expected from a consideration of the combined curves (4), (5) and (6), and this may be the result of a prolonged induction period. Evidence of induction periods has been obtained in earlier work on chlorophyll at high oxygen pressures,[13] and such periods are indeed characteristic of autoxidation reactions in general.[39]

Carotenoid Fraction (on thallous bromide)

Experimental. 1·23 mg. carotene

0·3 g. thallous bromide

Gauge sensitivity = 0·020 mm./scale division.

The following rates were obtained from two successive illuminations of the same film, interrupted by a period in vacuo. Initial oxygen pressures were (1) 3·2 div. and (2) 2·0 div.

	p	R_{100}	p	R_{100}		p	R_{100}	p	R_{100}
(1)	2·85	1·20	1·6	0·60	(2)	1·8	0·57	0·95	0·33
	2·4	0·97	1·25	0·50		1·4	0·47	0·8	0·27
	2·0	0·77	0·85	0·33		1·15	0·37	0·6	0·20

Absolute rates in this experiment are greater than with chlorophyll, but since the overall pressure changes in each illumination are small, the concentration of pigment may be taken as approximately constant; rates should then depend only on the oxygen pressure.

Graph 3 shows that the curve obtained from run (1) is nearly linear, and tends to a limit slightly above zero pressure. In run (2) an acceleration period is evident, but later rates coincide with those of (1). The extrapolation of the linear portion of the combined curves cuts the pressure axis at 0·1 div. ≡ 0·002 mm., which would appear to be the minimum 'dissociation' pressure for the oxide of carotene at 25°C.

Previous work on carotene at high oxygen pressures has shown[13] that its photo-oxidation is 'reversible', but only to a small extent, conversion of the oxide to its irreversible form being comparatively rapid. The small equilibrium pressure deduced for the present conditions appears to agree with this, and to bear out the previous conclusion that the value of 0·056 mm. pressure obtained with the chlorophyll—carotenoid mixture was due mainly to the chlorophyll.

The Effect of Carbon Dioxide

From the general viewpoint of photosynthesis, it was of interest to try the effect of carbon dioxide and water vapour on

the results obtained with chlorophyll at low oxygen pressures. Other aspects of the photo-oxidation, such as the molecular pressure decrease to pigment ratio and the 'reversibility', have been shown to be unaffected by the presence of these gases.[40] Accordingly the low pressure oxidation of the chlorophyll fraction was investigated in the presence of a constant amount of carbon dioxide.

Experimental. 5·29 mg. chlorophyll (copper-free sample)

0·3 g. thallous bromide

Gauge sensitivity = 0·020 mm./scale division.

Initial oxygen pressures taken were (1) 9·8 div., (2) 6·1 div. and (3) 4·2 div. After the addition of oxygen in each case, carbon dioxide was added to a pressure of 40 mm. and the film illuminated. Between runs the system was evacuated as before.

	p	R_{100}	p	R_{100}		p	R_{100}	p	R_{100}
(1)	9·0	0·42	5·0	0·50	(3)	3·1	0·40	2·6	0·125
	8·5	0·33	4·6	0·35		2·95	0·28	2·5	0·125
	8·0	0·32	4·3	0·25		2·8	0·18	2·4	0·125
(2)	5·5	0·68	4·1	0·16		2·7	0·13		

Measurement of the low rates involved was found to be more difficult at a total gas pressure of 40 mm. than in the previous experiments at less than 0·5 mm., due to the more appreciable effect of any slight fluctuations in temperature. This may account to some extent for the differences apparent between Graph 2 and the present results shown on Graph 4. Reaction curve (2) is approximately linear and appears to be tending to a limit of 0·074 mm. pressure. Evacuation and re-illumination at a lower initial oxygen pressure shows the rate to be restored,

and the curve obtained is similar in shape to that found near the critical limit. The extrapolated value in this case is 0·050 mm., and the corrected limit 0·054 mm. This agrees reasonably well with the limit from Graph 2 (0·052 mm.) and it thus appears that the presence of carbon dioxide has no appreciable effect on the value of the 'dissociation' pressure of photo-oxygenated chlorophyll.

If the product of chlorophyll photo-oxidation is a hydroperoxide, then the addition of carbon dioxide might result in absorption with the formation of a percarbonate thus -

$$R.COOH + CO_2 \longrightarrow RO.CO.OOH$$

by analogy with the reaction which takes place between carbon dioxide and inorganic peroxides, and this should give a change in the equilibrium pressure. No such effect would seem probable in the presence of water vapour, since decarboxylation of organic acids occurs readily under acid conditions. By making the medium alkaline, however, carboxylation should proceed more easily, the reaction then being ionic,[41] and in this connection the effect of carbon dioxide was further investigated in the presence of disodium orthophosphate. A similar effect has been studied at high oxygen pressures using ammonia vapour as the alkaline reagent;[42] no definite uptake of carbon dioxide was detected.

Experimental. 2·62 mg. chlorophyll (copper-free sample)

0·3 g. thallous bromide

1 ml. disodium orthophosphate solution (1·056 g./litre in water)

Gauge sensitivity = 0·020 mm./scale division.

The solution of the phosphate was added to the film of chlorophyll on thallous bromide, made in the normal way, and the water removed

at the oil pump over a trap of phosphoric oxide. The amount taken was such as to give a molecular ratio of pigment to phosphate of unity.

(a) The film was illuminated at an initial oxygen pressure of 25 div. (0·5 mm.), until a pressure decrease of 2·5 div. had occurred. The oxygen was then pumped off quickly in the dark and carbon dioxide (5 div.) rapidly added. A small increase in pressure in the dark was observed of approximately 0·5 div., a steady state being reached which was not altered by the further addition of carbon dioxide or by illumination. Since no absorption was recorded, it must be assumed that no carboxylation had taken place under these conditions.

(b) After evacuation, oxygen was added to a pressure of 5 div. (0·1 mm.), the film illuminated, and pressure readings taken at intervals over several hours. Illumination was then stopped while 20 div. carbon dioxide were added, and recommenced when steady conditions were reached. The rate curve obtained was approximately linear, rates found after the addition of the carbon dioxide lying on the extrapolation of the initial portion of the curve. The corrected limit obtained from the complete curve was 0·053 mm. which was in good agreement with the value for chlorophyll in the presence of oxygen alone.

(c) After evacuation the film was illuminated in oxygen as in (a), until a pressure decrease of 3 div. had occurred. Removal of the remaining gas by evacuation in the dark, was followed by the addition first of approximately 5 mm. water vapour, and then of 30 div. carbon dioxide when equilibrium had been reached. A slow decrease in pressure in the dark was observed, the total change over a long period being approximately 3 div. Illumination

when a steady pressure had been reached, produced no further change. Repetition of this procedure showed that the results obtained were not reproducible, a decrease in pressure of less than 0·5 div. being recorded on the addition of carbon dioxide, and a similar increase on illumination. At the pressure of mixed vapours concerned, such small effects might be due in part to unavoidable temperature fluctuations.

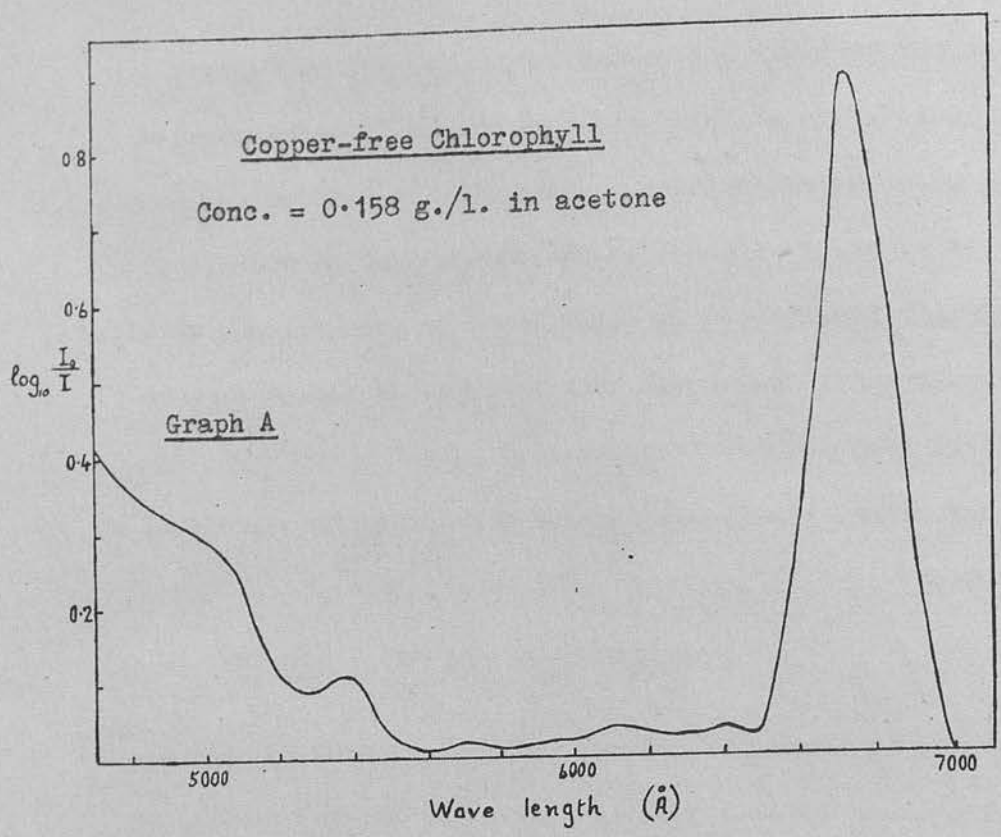
Since primary phosphate absorbs carbon dioxide according to the equation[43]

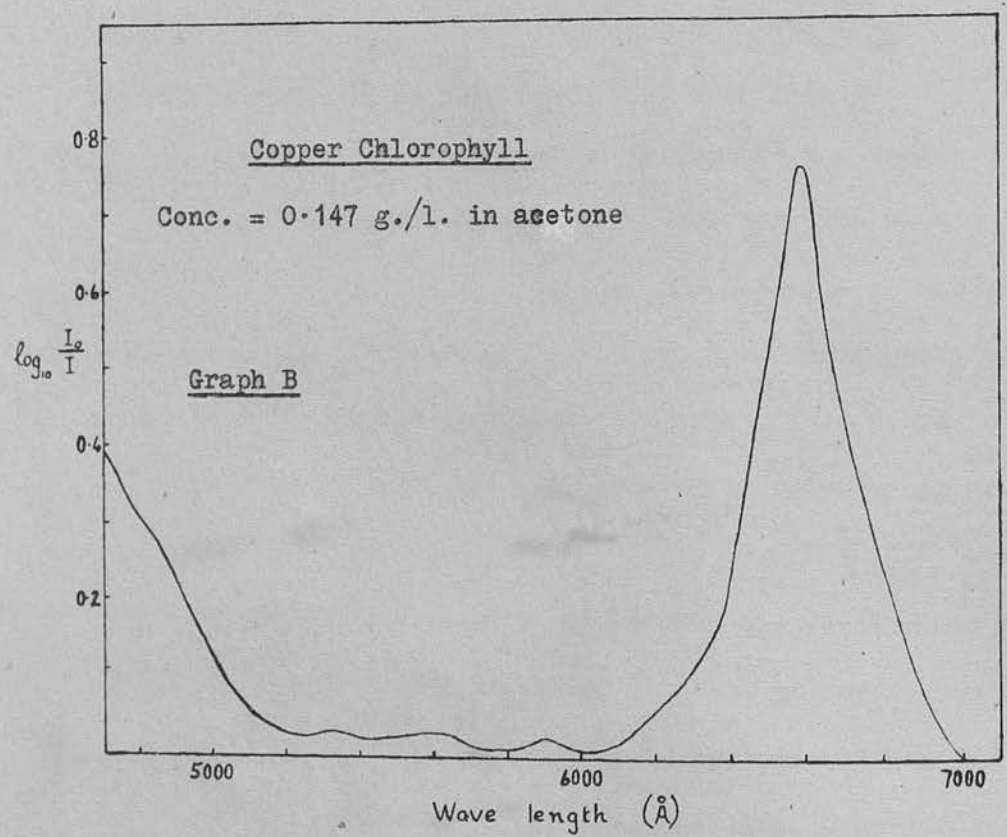
$$CO_2 + H_2O + HPO_4^{--} \rightleftharpoons HCO_3^- + H_2PO_4^-$$

the decrease in pressure observed on adding carbon dioxide the first time under these conditions may have been due partly, or altogether, to this, and not to the presence of the oxide of chlorophyll. In view of the irreproducibility observed in this experiment with water vapour, it was not considered practicable to further this investigation by a comparison of the effects observed in the presence and absence of the oxide of chlorophyll. Accordingly no definite conclusion may be drawn from the observations under these conditions.

The gases present at the end of this experiment were frozen out in a liquid oxygen trap and tested for peroxide and aldehyde reactions[14] as described previously (p. 26). The tests proved negative.

In summarising the experiments with carbon dioxide it may be said that, even in alkaline conditions, no definite uptake of carbon dioxide by chlorophyll or its oxide has been detected, while the critical limit for this sample of chlorophyll is unaffected by the presence of the gas.

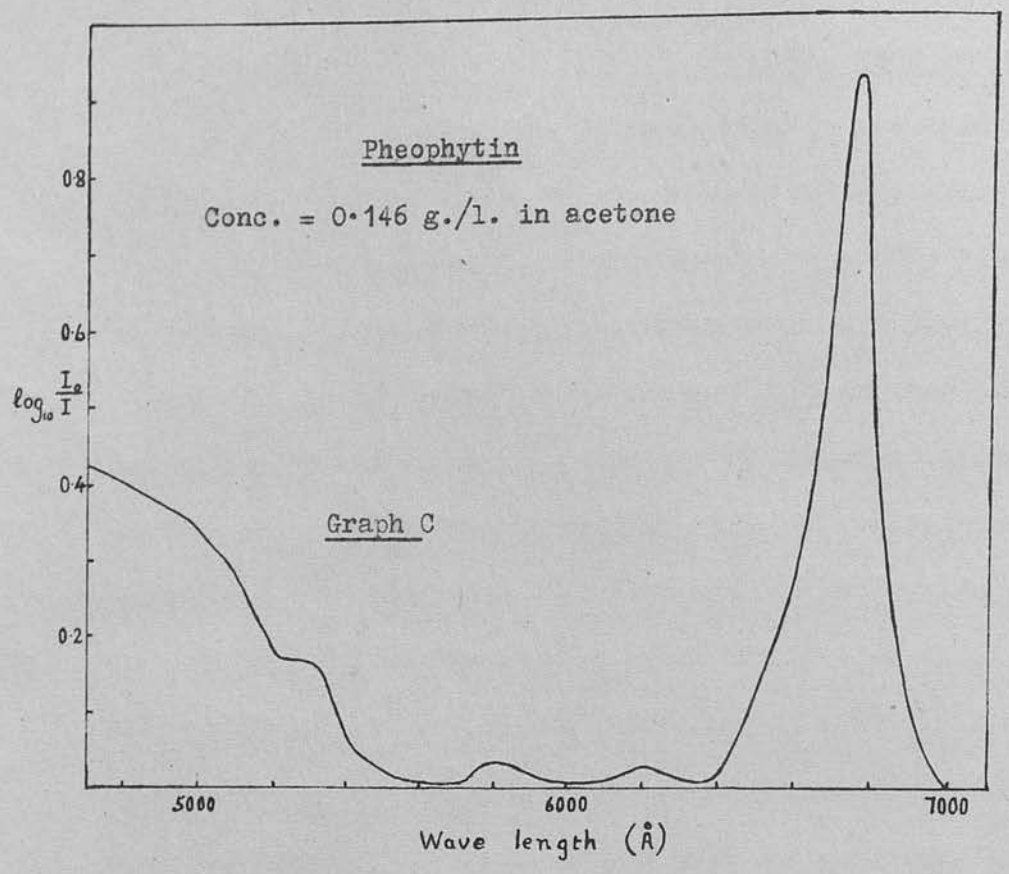

Copper-free Chlorophyll
Conc. = 0·158 g./l. in acetone
Graph A
0·8
0·6
0·4
0·2
$\log_{10} \frac{I_0}{I}$
5000
6000
7000
Wave length (Å)

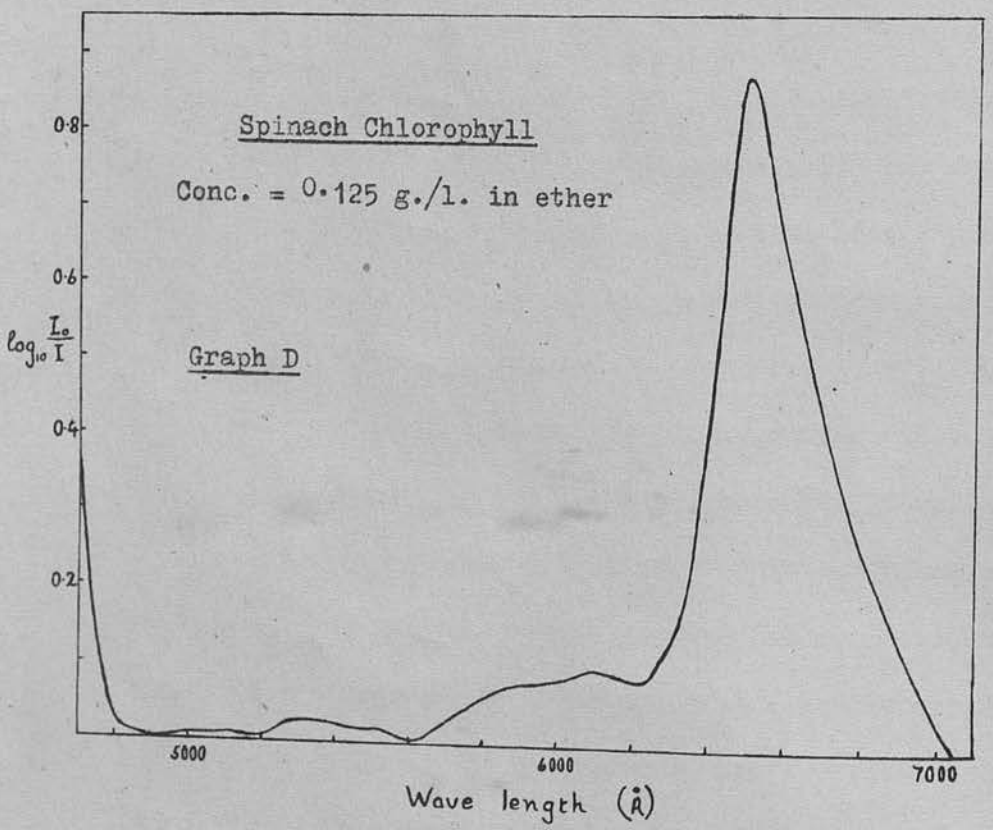

Copper Chlorophyll
Conc. = 0·147 g./l. in acetone
Graph B
0·8
0·6
0·4
0·2
$\log_{10} \frac{I_0}{I}$
5000
6000
7000
Wave length (Å)

Copper-free Chlorophyll

Examination of the absorption spectrum of the copper-free chlorophyll solution used in the previous experiments, showed a maximum at 6700 - 6750 Å and a band in the region of 5300 - 5400 Å (Graph A). By a comparison with the spectra reported by Zscheile[27] and Comar for pure chlorophyll and for pheophytin-chlorophyll mixtures, it appeared that the solution must contain some pheophytin. Results obtained at this stage in the present investigations, from work carried out on a different aspect of the oxidation of chlorophyll films on thallous bromide[13c], confirmed this observation. Thus it was found that whereas pure chlorophyll had a molecular pressure decrease ratio of unity and pheophytin a ratio of three, in the case of this copper-free sample the ratio observed was approximately two. The presence of approximately 50% pheophytin in the copper-free solution would explain this ratio, and would account for the pheophytin absorption characteristics evident in the spectrum. The positive phase test and bright red fluorescence in ultra violet light shown by this sample, are reactions characteristic of both chlorophyll and pheophytin. Pheophytin is a common impurity in preparations of chlorophyll, and separation of the two compounds is very difficult because of the marked similarity between them.[27]

In view of these observations, the question arises as to which molecule the 'dissociation' pressure observed with this sample applies, to the pure chlorophyll having one assumed oxidisable position in the molecule, as evidenced by its unit molecular pressure decrease ratio, or to the magnesium-free chlorophyll or pheophytin having three oxidisable positions,


Pheophytin
Conc. = 0·146 g./l. in acetone
Graph C
0·8
0·6
0·4
0·2
$\log_{10}\frac{I_o}{I}$
5000
6000
7000
Wave length (Å)


Spinach Chlorophyll
Conc. = 0·125 g./l. in ether
Graph D
0·8
0·6
0·4
0·2
$\log_{10}\frac{I_o}{I}$
5000
6000
7000
Wave length (Å)

since the oxidation of both compounds has been shown to be 'reversible' to a certain extent, less so with pheophytin than with chlorophyll. Accordingly the low pressure oxidations were investigated, first of the copper-containing chlorophyll sample, then of pheophytin, and finally of pure chlorophyll extracted directly from spinach leaves.

Copper Chlorophyll

The acetone solution of the bonemeal separated copper-containing chlorophyll was a clear green colour in contrast to the olive green of the copper-free sample. Its absorption spectrum (Graph B) showed a maximum in the red at 6655 - 6600 Å, and agreed reasonably well with that reported by Rabinowitch and Weiss[11] for pure chlorophyll. As mentioned earlier (p. 20), the non-occurrence of the phase reaction and fluorescence with this solution has been attributed to the effect of the copper present.

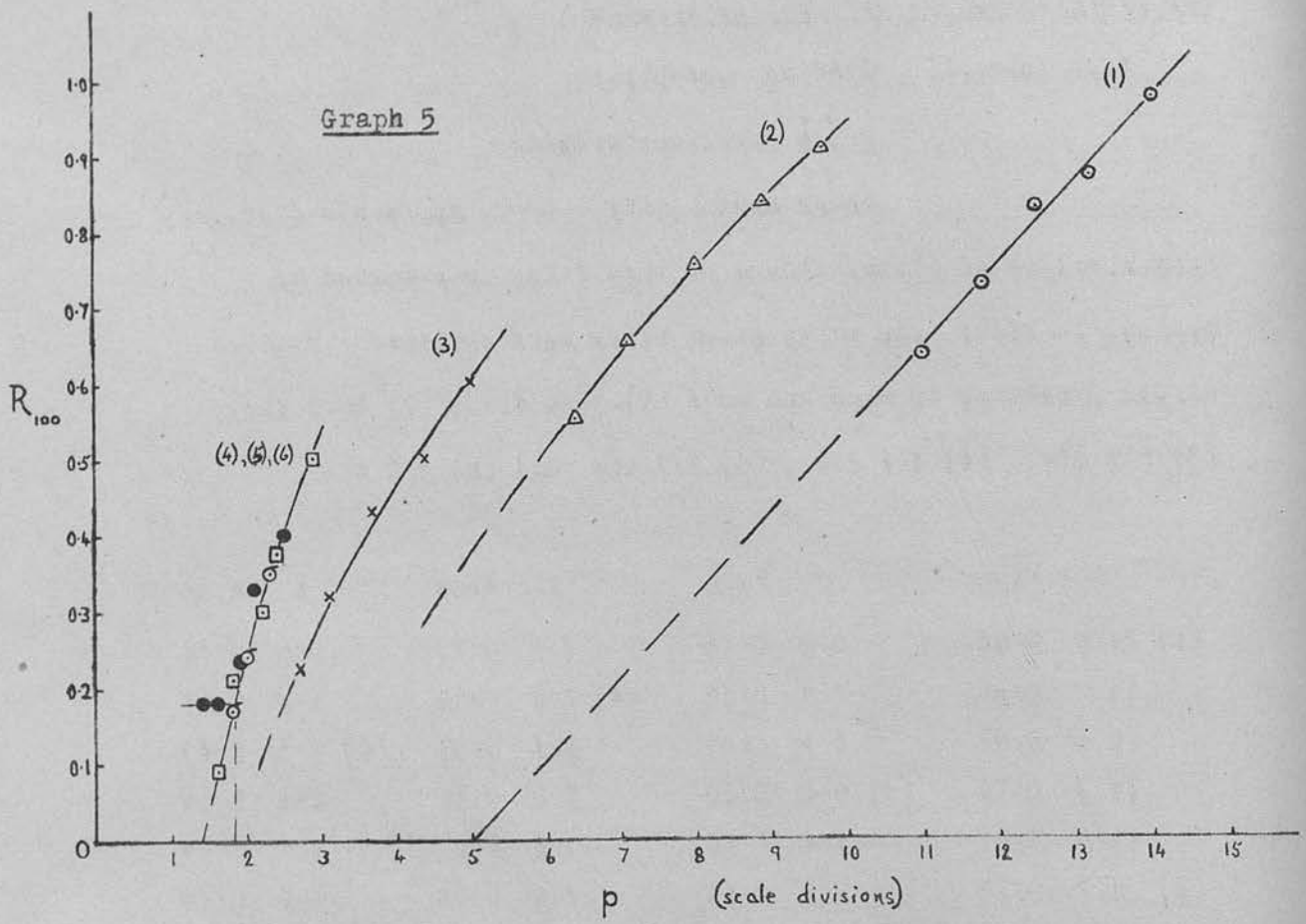
It was found that rates obtained in the pressure region below 0·5 mm. oxygen with this sample of chlorophyll deposited on thallous bromide, were too low to be measured accurately (being less than one tenth of the previous rates), and so the dissociation pressure of any reversible oxide could not be found.

Pheophytin

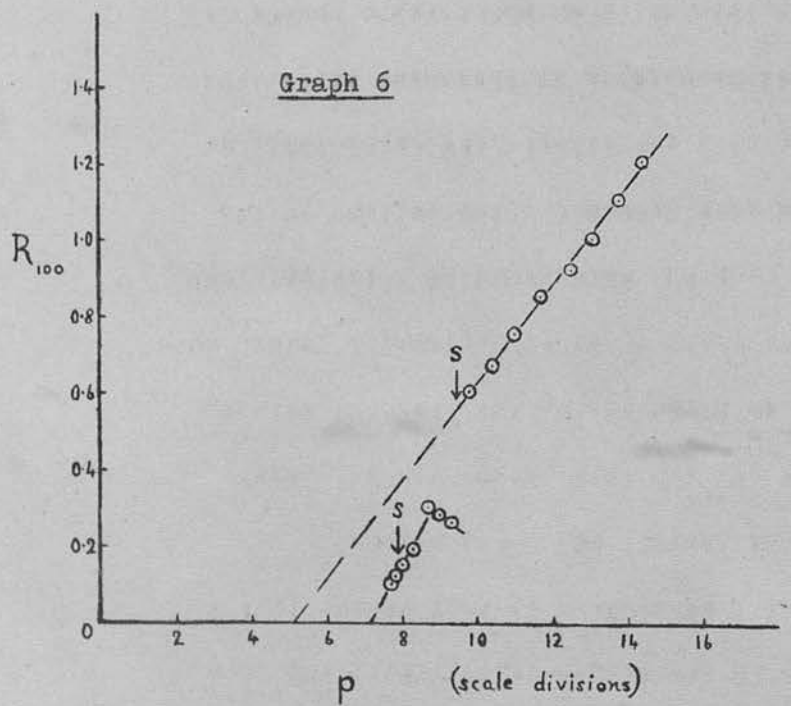
Pure pheophytin was prepared from the copper-free chlorophyll solution as described on page 21. Its solution in acetone fluoresced and gave the phase test, and its absorption spectrum (Graph C) agreed satisfactorily with that reported by Zscheile and Comar[27]. Rates in the low pressure region with this preparation were found to be measurable, and its oxidation was therefore examined in the same way as that of the copper-free chlorophyll.

Pheophytin (on thallous bromide)

Graph 5



Graph 6



The photo-oxidation on thallous bromide

Experimental. 2·42 mg. pheophytin

0·3 g. thallous bromide

Gauge sensitivity = 0·020 mm./scale division.

From a series of illuminations of this film, interrupted by periods in vacuo, the rates given below were obtained. Initial oxygen pressures in each run were (1) 15·0 div., (2) 10·2 div., (3) 5·8 div., (4) 3·4 div., (5) 2·7 div. and (6) 3·0 div.

	p	R_{100}		p	R_{100}		p	R_{100}		p	R_{100}
(1)	14·0	0·96		8·0	0·75		2·7	0·22		2·0	0·24
	13·2	0·86		7·1	0·65	(4)	2·9	0·50		1·8	0·17
	12·5	0·82		6·4	0·55		2·4	0·37	(6)	2·5	0·40
	11·8	0·72	(3)	5·0	0·60		2·2	0·30		2·1	0·33
	11·0	0·63		4·4	0·50		1·8	0·21		1·9	0·23
(2)	9·7	0·90		3·7	0·43		1·6	0·09		1·6	0·18
	8·9	0·83		3·1	0·32	(5)	2·3	0·35		1·4	0·18

In this experiment with pheophytin an appreciable change in rate of pressure decrease was measurable at pressures below that of the critical limit (0·052 mm.) for copper-free chlorophyll at the same temperature. Rates from separate illuminations in the pressure region below about 0·06 mm. were found to coincide: thus from runs (4), (5) and (6) of Graph 5, an approximately linear rate curve was obtained, which when produced cut the pressure axis at 1·4 div. ≡ 0·028 mm. In run (6) the rate became almost steady (R_{100} = 0·18) after decreasing rapidly as in (4) and (5). By comparison with the chlorophyll experiment it will appear that a correction should be applied to the extrapolated limit, and from Graph 5 the corrected limit is seen to be 0·036 mm.

At higher initial oxygen pressures, rates were lower than might be expected from this combined curve, but in each run the rates obtained appeared to be tending to limiting oxygen pressures which approached the final limit. Re-illumination of the film after run (6) at an initial oxygen pressure equal to that of run (1), showed that the original rate of oxidation was restored. From this it can be assumed that the overall decrease in rate observed during the series of illuminations (1) to (4), has not been due to the simultaneous decrease in pheophytin concentration causing a decreased I_{abs}.

Repetition of this experiment with another film of pheophytin on thallous bromide confirmed the following general points -

(a) The extrapolated limit of 0·028 mm. was reproduced. No further evidence of the steady rate observed in run (6) was obtained; instead, rates decreased rapidly as in run (4) to the limiting pressure. In view of the 'reversibility' observed in experiments at high oxygen pressures this would appear to be the dissociation pressure for pheophytin at 25°C. Since this limit is below the value of 0·052 mm. obtained for chlorophyll-pheophytin, it would appear that the 0·052 mm. is due to chlorophyll itself.

13c

(b) At pressures considerably above this limit, rates were much lower than might be expected, and decreased during illumination more rapidly than can be accounted for solely by the simultaneous decrease in pheophytin concentration. Evacuation appeared to restore such decreased rates, and resulted in different rates being obtained for the same pressure.

(c) Reproducible rates were only obtained after long illumination and at pressures near the critical limit.

These points agree with the general observations made in the similar examination of the copper-free chlorophyll solution.

A possible explanation of (b) is that oxidation is accompanied by the production of some retarding product or reaction and that removal of this by evacuation results in the apparent increase in rate (cf. p. 5). In this connection, a film of pheophytin was illuminated at 100 mm. oxygen pressure until an appreciable decrease in pressure (0·6 mm.) had been observed. The gases present then were frozen out and tested for hydrogen peroxide as described previously (p. 26). All such tests proved negative.

It was possible that run (1) if continued sufficiently long, would give a rate curve which would reach the limit of 0·028 mm. Accordingly another film of the same amount of pheophytin on thallous bromide was illuminated at an initial oxygen pressure of 15·2 div.; instead of evacuating after illumination as before, it was allowed to stand in the dark for several hours at the pressure of gas remaining at the time the light was cut off. No change in pressure of the system was observed to have occurred during this dark period. On re-illumination the rate of oxidation was found, however, to have decreased considerably. Rates obtained were as follows -

p	R_{100}	p	R_{100}	p	R_{100}	p	R_{100}
14·4	1·20	11·7	0·85	9·3	0·26	8·0	0·15
13·8	1·10	11·0	0·75	9·0	0·28	7·8	0·12
13·1	1·00	10·4	0·67	8·7	0·30	7·7	0·10
12·5	0·92	9·8	0·60	8·3	0·19		

Positions marked S on Graph 6 denote dark periods of approximately 12 hours at the pressures indicated. The rate curve obtained on

first illuminating the film, appears to be tending to the same limit as curve (1) of Graph 5, that is to a pressure considerably greater than the critical limit of 0·028 mm. If this is due to some retarding reaction taking place during oxidation, then the decrease in rate observed after the dark period in oxygen may be the result of its continuation in the dark. The effect seems therefore to be partly, if not entirely, thermal. Evacuation of the system after the complete run shown on Graph 6, and re-illumination at an oxygen pressure of 15 div., showed a return of the rate to approximately its original value. Thus in general we have a retardation of rate on standing in oxygen and the removal of this retardation by evacuation.

Repetition of this procedure of illuminating, standing in the dark in oxygen, and re-illuminating, at a later stage in the oxidation of the same film and at the same initial oxygen pressure, showed a decrease in rate after standing which was much less marked than in the first instance. The retarding effect, therefore, appears to be greatest in the initial illumination of the pigment.

The photo-oxidation on glass

Since the behaviour of pheophytin on illumination in oxygen may depend to some extent on the nature of the sensitising substrate used, a similar examination was made of the pigment when deposited on powdered glass, instead of on thallous bromide.

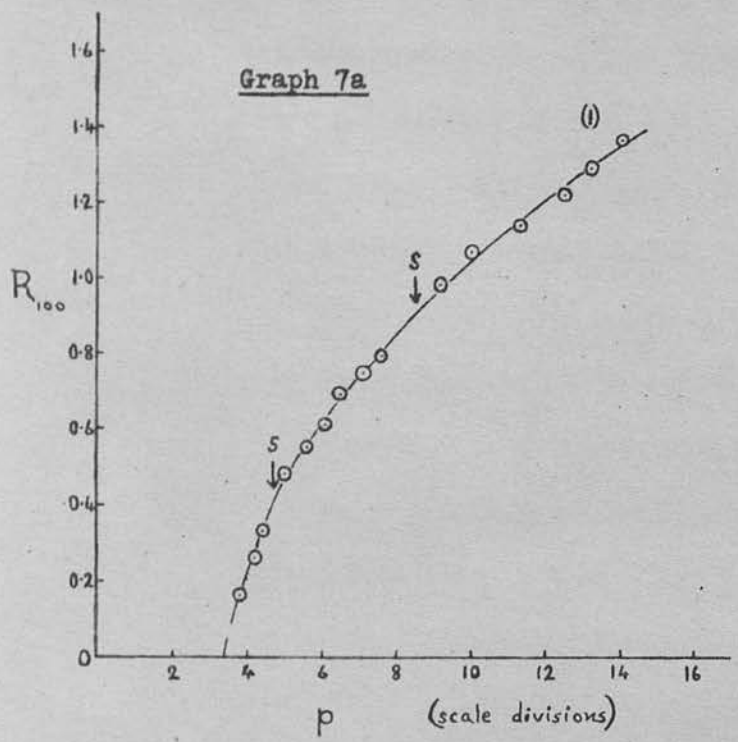
Experimental. 2·18 mg. pheophytin

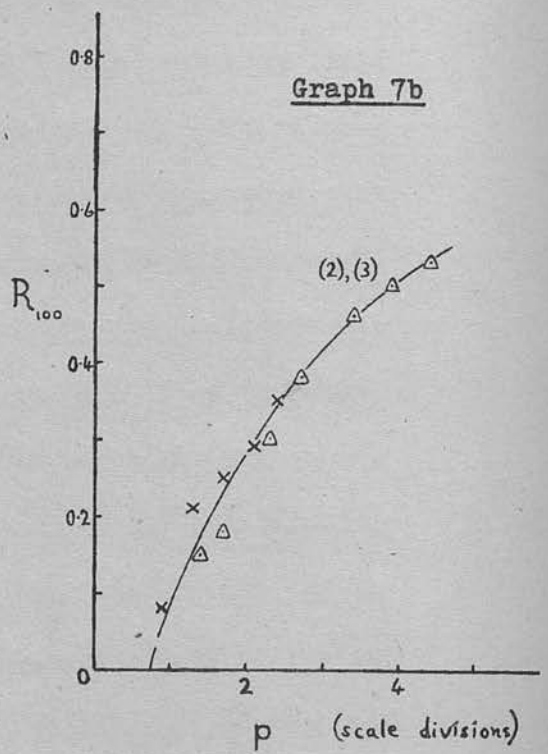
0·3 g. powdered glass (Jena)

Gauge sensitivity = 0·020 mm./scale division.

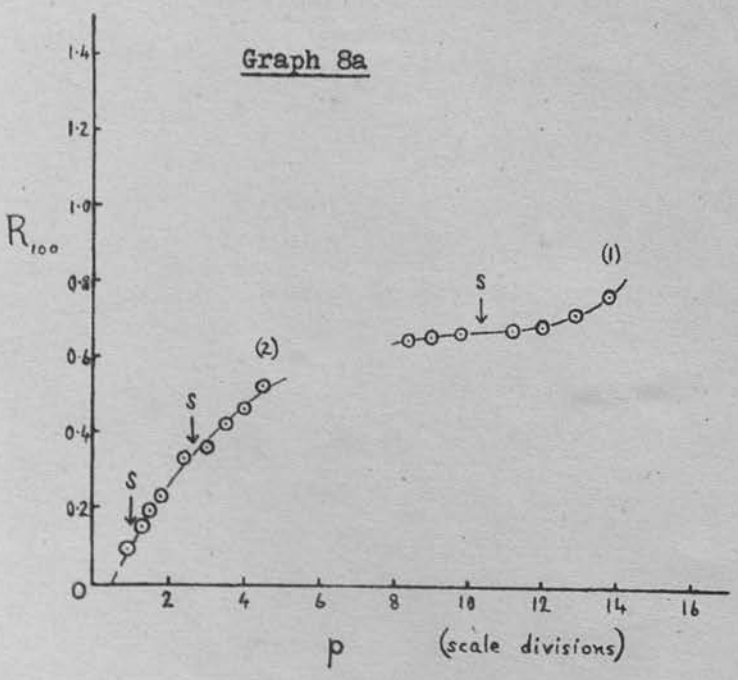
Initial oxygen pressures taken were (1) 14·2 div., (2) 5·0 div. and (3) 2·9 div.

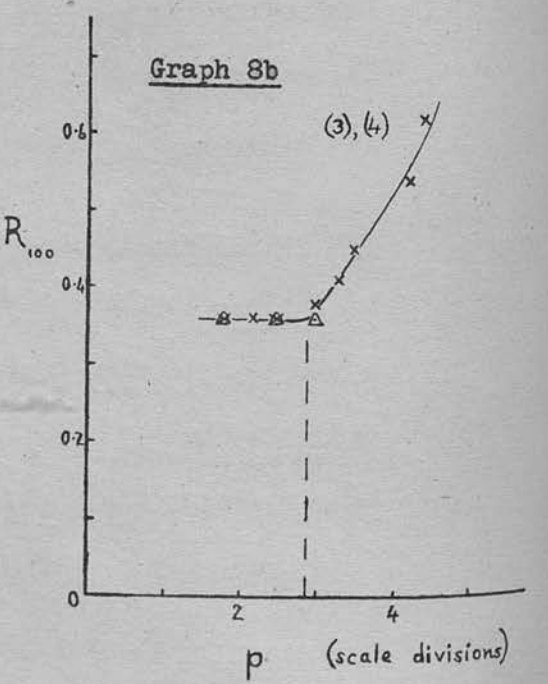
Pheophytin (on glass)





Copper-free Chlorophyll (on glass)





	p	R_{100}	p	R_{100}		p	R_{100}		p	R_{100}
(1)	14·0	1·36	6·5	0·70	(2)	4·4	0·53	(3)	2·4	0·35
	13·2	1·29	6·1	0·62		3·9	0·50		2·1	0·29
	12·5	1·22	5·6	0·56		3·4	0·46		1·7	0·25
	11·3	1·14	5·0	0·49		2·7	0·38		1·3	0·21
	10·0	1·07	4·4	0·34		2·3	0·30		0·9	0·08
	9·2	0·99	4·2	0·27		1·7	0·18			
	7·6	0·80	3·8	0·17		1·4	0·15			
	7·1	0·75								

The rate curve (1) of Graph 7a represents an illumination of pheophytin interrupted only by dark periods in oxygen at the pressures marked S. No marked retardation of rate is evident after such intervals in this case, and the curve may thus be taken as continuous. During this run the total pressure decrease is considerable (11·5 div.) and the fall off in rate observed must be due in part to the corresponding change in pheophytin concentration. Such was shown to be the case by a re-illumination at 15 div. oxygen pressure, after evacuation, when rates were found to be only two thirds of the original rates at the same pressures. The limit of 0·068 mm. obtained by extrapolation of this curve will consequently be higher than the true limit for pheophytin. Rates from runs (2) and (3) of Graph 7b, where the overall pressure change was small, were found to coincide, and extrapolation of the combined curve gave zero rate of oxidation at 0·015 mm.

In comparing oxidations of pheophytin on glass and on thallous bromide films generally, it may be noted that at the low oxygen pressures concerned, the rates in each case are of the same order. This is in contrast to the observation made previously[13] at high oxygen pressures (100 mm.) that on thallous

bromide corresponding rates were very much greater. This acceleration effect was observed in 'white' light but not in red, the latter being absorbed by the pigment but not by the thallous bromide, and has been attributed to a sensitisation of the reaction by the thallous bromide.

The main differences evident between the results for pheophytin on glass and on thallous bromide, are the lower limit obtained on glass, i.e. 0·015 mm. as compared to 0·028 mm., and the absence of any obvious retardation in rate after dark periods in oxygen on glass. The anomalous appearance of different rates for the same gas pressures, noted previously with thallous bromide films after evacuation, is however apparent on glass also, greater rates being observed in run (2) after evacuation than at the same pressures in run (1).

Thallous Bromide

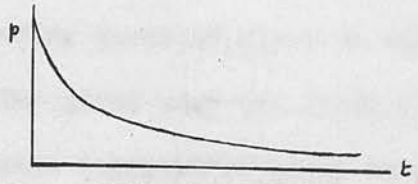
In an attempt to elucidate some of the differences apparent between results obtained using glass as substrate and those using thallous bromide, the effect of oxygen on thallous bromide crystals alone was re-investigated. This has been examined by previous investigators[13] at 100 mm. oxygen pressure, and no reaction has been found to occur. It was possible that under the conditions of the above experiments, involving a more sensitive gauge, some reaction might be detectable.

Experimental. 0·3 g. thallous bromide

Gauge sensitivity = 0·020 mm./scale division.

The film was prepared in the normal way, a few drops of acetone being used to spread out the thallous bromide. No change in pressure was evident with it in the dark or on illumination in

vacuo. On the addition of oxygen (0·3 mm.) no change occurred in the dark, but on illumination a small but appreciable decrease in pressure was observed. The rate of decrease fell off rapidly, giving a pressure-time curve of the form



Evacuation followed by re-illumination in oxygen, produced an increase in the rate of oxidation, while standing for several hours in the dark in oxygen resulted in a decrease in rate. The same general effect was observed using different samples of thallous bromide at different initial oxygen pressures in the region below 1 mm. The actual pressure decrease observed on illumination, and the rate at which this decrease occurred, varied with the film. At initial oxygen pressures below 0·5 mm. (25 div.) the maximum decrease observed on first illuminating a sample, for a period similar to that employed in the previous experiments, was of the order of 0·05 mm. (2-3 div.). Subsequent illuminations of the same film after evacuation, resulted in successively decreasing pressure changes over similar periods of time.

At 100 mm. pressure a decrease of approximately 0·03 mm. was obtained during the first few hours illumination, and thereafter the pressure remained almost steady.

Since the extent of the reaction observed here at low pressures is appreciable in relation to the magnitude of the pressure changes obtained in the previous experiments, some allowance must obviously be made for such an effect in the consideration of these results. In view, however, of the irreproducible nature of the effect with different experimental

preparations, and in consideration of the fact that it must be modified to a large extent by the presence of a covering layer of pigment, an exact correction is not possible. The low pressure oxidation of the copper-free chlorophyll sample was, therefore, repeated using powdered glass as substrate instead of thallous bromide. The glass used was first confirmed to involve no pressure change when illuminated alone in oxygen under similar conditions.

Copper-free Chlorophyll on Glass

Experimental. 2·25 mg. chlorophyll (copper-free sample)
0·3 g. powdered glass (Winchester)
Gauge sensitivity = 0·020 mm./scale division.

The rates obtained from a series of illuminations of this film at the following initial oxygen pressures are given below - (1) 14·5 div., (2) 5·0 div., (3) 3·5 div. and (4) 5·0 div.

	p	R_{100}		p	R_{100}		p	R_{100}	p	R_{100}
(1)	13·8	0·76	(2)	4·5	0·52		1·3	0·15	3·5	0·45
	12·9	0·71		4·0	0·46		0·9	0·09	3·3	0·41
	12·0	0·68		3·5	0·42	(3)	3·0	0·36	3·0	0·38
	11·2	0·67		3·0	0·36		2·5	0·36	2·5	0·36
	9·8	0·66		2·4	0·33		1·8	0·36	2·2	0·36
	9·0	0·65		1·8	0·23	(4)	4·4	0·62	1·8	0·36
	8·4	0·64		1·5	0·19		4·2	0·54		

At the positions marked S on Graph 8a, illumination was interrupted by dark periods in oxygen at the pressures indicated. Otherwise runs were followed as before by evacuation to a pressure of less than 0·001 mm.

In comparing the results obtained with the corresponding ones

on thallous bromide (Graph 2), the following general points may be noted.

(a) Rates are of the same order as on thallous bromide.

(b) No retardation of rate is evident after dark periods in oxygen.

(c) The 'dissociation' pressure limit obtained by extrapolation of curve (2) is lower than that found on thallous bromide, the value here being 0·01 mm. as compared to the previous value of 0·052 mm.

These observations are in agreement with the results obtained in the corresponding experiments on pheophytin.

Re-illumination of the film after run (2), in the pressure region below 0·1 mm., showed a restoration of rate to almost the same value as before. In these runs, (3) and (4), of Graph 8b, it was found that below about 3 div. pressure, the rate of pressure decrease became almost constant. If this constant rate represents the direct conversion of chlorophyll into stable oxide, as well as the oxidation of contaminant pheophytin, then the pressure at which the two portions of the curve intersect (cf. p. 34) may be taken as the corrected critical limit for chlorophyll. The limit thus obtained is 0·058 mm., a value slightly higher than that found for the same sample of chlorophyll when deposited on thallous bromide.

The reason for the apparent limit of curve (2), and its disagreement with that from runs (3) and (4), is not immediately evident, but it may be connected in some way with the fact that copper-free chlorophyll is a mixture, one of the components of which has more than one oxidisable position in its molecule.

The photo-oxidation on thallous iodide

A similar examination was made of the copper-free chlorophyll sample when deposited on thallous iodide. This compound has been shown[13] to have the same accelerating effect on the rate of photo-oxidation of chlorophyll at high pressures as has the bromide, and it was therefore of interest to see whether the general features observed in the low pressure region with it as substrate were the same as with thallous bromide.

The results obtained may be summarised as follows -

(a) A very small decrease in pressure was observed on the illumination of the thallous iodide alone at pressures below 0·3 mm. oxygen. The effect was similar to, but less appreciable than, that formed with thallous bromide.

(b) Rates with copper-free chlorophyll deposited on the crystals, were of the same order as those found using thallous bromide or glass as substrate at corresponding oxygen pressures.

(c) A decrease in rate was observed after dark periods in oxygen, the retardation being removed by evacuation.

(d) As in all previous experiments, the anomaly of different rates for the same pressures in separate illuminations of the same sample was observed, the differences arising as before during later oxidations when the rates observed were greater than those of the initial illuminations.

(e) After the first few illuminations, rate points from separate illuminations in the pressure region below approximately 0·1 mm. were found to coincide and gave an extrapolated limit of 0·024 mm. The corrected limit, obtained by allowing for the rate of conversion of reversible to stable oxide, was 0·040 mm. at 25°C.

This value is somewhat lower than the corresponding one observed using thallous bromide as substrate.

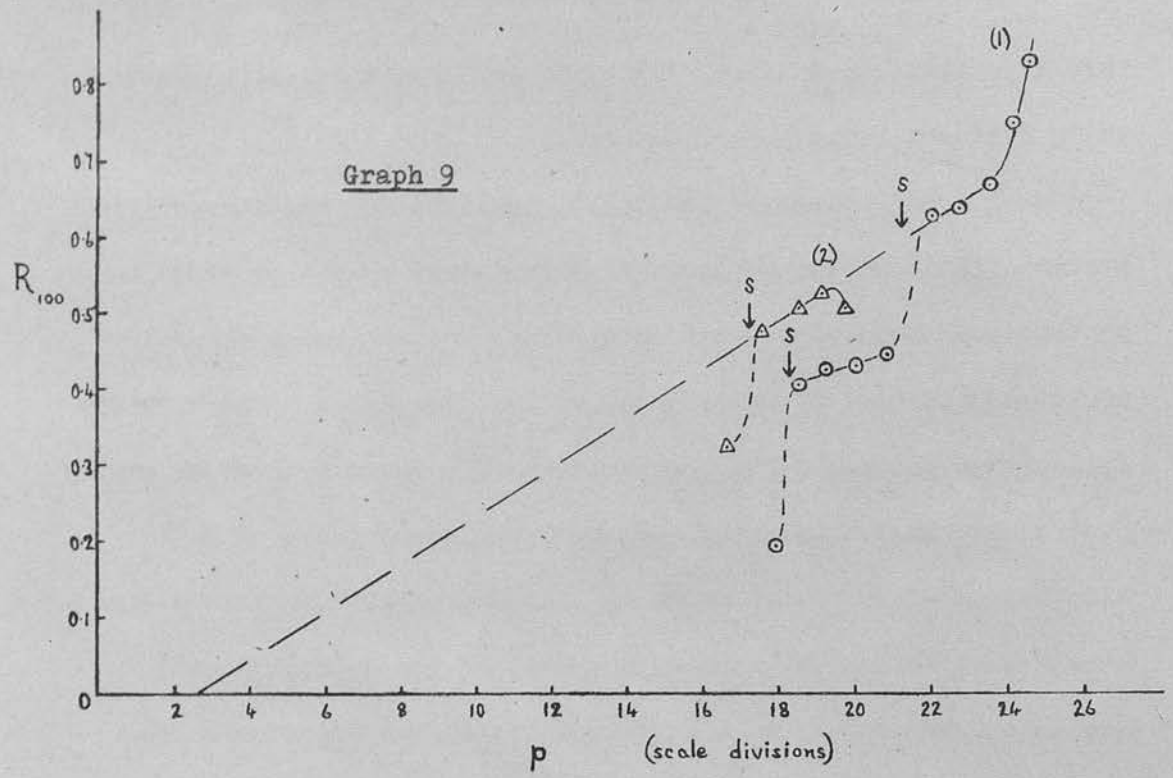
From a comparison of the values obtained for the 'dissociation' pressure limit of the copper-free chlorophyll sample on glass and on thallous iodide with that obtained in the earlier examination of the same sample of chlorophyll on thallous bromide, it appeared that either the value was dependent on the substrate used or else some change in the solution had occurred. Repetition of the examination of the same sample on thallous bromide at this stage, showed that though the general features of the oxidation were unchanged, the value for the critical limit had decreased. The extrapolated value obtained was the same as found on thallous iodide, namely 0·024 mm. The tendency observed here for the limit to approach that observed with pure pheophytin, appears to indicate that further degradation of the chlorophyll had occurred during the interval between these experiments.

In view of this, pure chlorophyll was prepared from spinach leaves and its dissociation pressure limit investigated immediately after preparation.

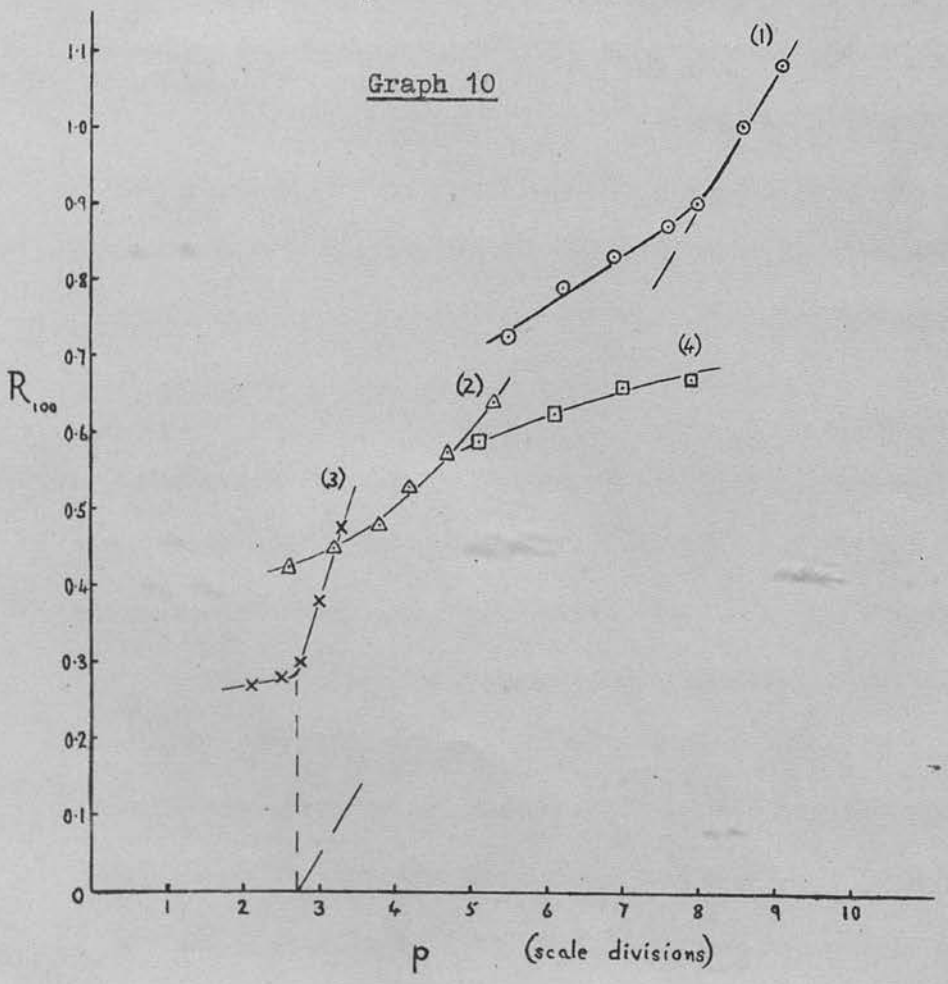
Spinach Chlorophyll

This chlorophyll sample was obtained by direct extraction from spinach leaves, as already described (p. 18). The absorption spectrum (Graph D) of the ether solution of the mixed chlorophylls a and b, after separation from carotenoids on sucrose, agreed satisfactorily with the results reported by Zscheile[27] and Comar and by Rabinowitch and Weiss.[11] The maximum in the red end of the spectrum lay near 6500 Å, and there was no evidence of pheophytin. The solution fluoresced red under ultra violet light and gave a

Spinach Chlorophyll (on thallous bromide)



Spinach Chlorophyll (on glass)



positive phase test. This chlorophyll preparation was much less stable than the copper-containing sample, and if kept in the solid state for several weeks was found to have undergone partial degradation to pheophytin, as shown by its absorption spectrum.

The photo-oxidation on thallous bromide

The low pressure oxidation of this sample was examined first when deposited on thallous bromide.

Experimental. 2·19 mg. spinach chlorophyll
0·3 g. thallous bromide
Gauge sensitivity = 0·020 mm./scale division.

Preliminary illuminations of this film at initial oxygen pressures of 0·3 mm., and less, showed the rate of pressure decrease to be smaller than that obtained under similar conditions with the copper-free sample of chlorophyll, but nevertheless appreciable in comparison to that with the copper-stabilised sample. At higher initial oxygen pressures - (1) 25·1 div. and (2) 20·0 div. - the following rates were obtained.

	p	R_{100}	p	R_{100}		p	R_{100}	p	R_{100}
(1)	24·5	0·82	22·0	0·62		18·5	0·40	18·5	0·50
	24·1	0·74	20·8	0·44		17·9	0·19	17·5	0·47
	23·5	0·66	20·0	0·42	(2)	19·7	0·50	16·6	0·32
	22·7	0·63	19·2	0·42		19·1	0·52		

The positions marked S on Graph 9 denote dark periods in oxygen at the pressures indicated. It is obvious from the graph that such treatment has resulted in a decrease in the rate of pressure change similar to that found previously (with pheophytin on thallous bromide and copper-free chlorophyll on thallous iodide),

although no corresponding change in the pressure of the system was observed. Evacuation after each run was found to remove this retarding effect as before.

Extrapolation of the rate curves obtained in runs (1) and (2) after evacuation and before these dark periods, shows that zero rate would be reached at a finite pressure of oxygen, and that the pressure concerned lies in the same region (i.e. approx. 0·054 mm.) as found with the copper-free chlorophyll. Owing to the difficulty experienced in measuring rates at lower pressures with this sample, a long extrapolation was necessary to obtain the critical limit, and accordingly the value found is only approximate.

It is possible at this stage to make a general comparison between the absolute rates observed with the three samples of chlorophyll at these low oxygen pressures under otherwise similar conditions. Thus with pure spinach chlorophyll as the standard, it is seen that the presence of copper has a retarding effect on the rate, while the removal of the central magnesium atom from the molecule results in an increased rate. This agrees with the general conclusions reached in work at high oxygen pressures. 13c

The photo-oxidation on glass

A similar examination of spinach chlorophyll on powdered glass showed that rates were of the same order as on thallous bromide. No retardation was observed after dark periods in oxygen as on thallous bromide, and in this respect the results resembled those found with copper-free chlorophyll and pheophytin on glass.

In view of the difficulty found in measuring the comparatively low rates involved, this experiment was repeated using the small

reaction vessel (see p. 14).

Experimental. 1·73 mg. spinach chlorophyll

0·2 g. powdered glass (Winchester)

Gauge sensitivity = 0·020 mm./scale division.

Initial oxygen pressures in the illumination of this film were (1) 10·0 div., (2) 5·8 div., (3) 3·7 div. and (4) 8·4 div. Each run was followed by evacuation and the rates obtained were as follows -

	p	R_{100}		p	R_{100}		p	R_{100}		p	R_{100}
(1)	9·1	1·08		5·5	0·725		2·60	0·425	(4)	7·9	0·670
	8·6	1·00	(2)	5·3	0·640	(3)	3·30	0·475		7·0	0·660
	8·0	0·90		4·7	0·575		3·00	0·380		6·1	0·625
	7·6	0·87		4·2	0·530		2·75	0·300		5·1	0·590
	6·9	0·83		3·8	0·480		2·50	0·280			
	6·2	0·79		3·2	0·450		2·10	0·270			

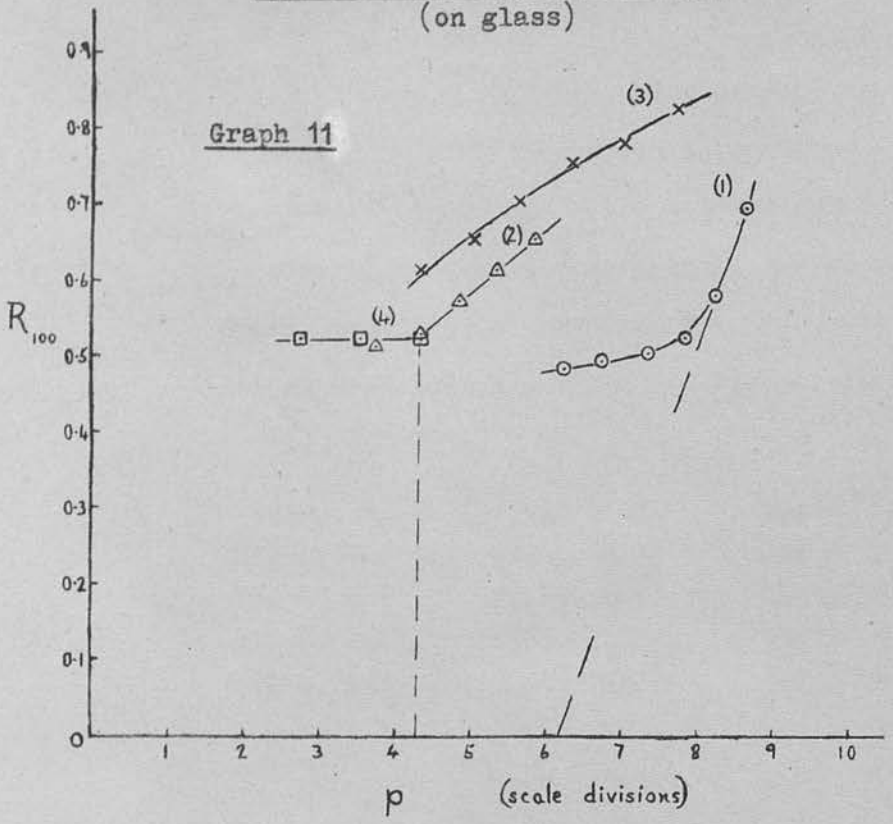
In general one would expect that the magnitude of the observed rates of formation of reversible chlorophyll oxide and of the final irreversible form, would both be increased by the use of the smaller vessel. The form of the rate curve in the region of the dissociation pressure (p. 34) should remain essentially the same, however, and this is seen to be the case here (Graph 10). Thus in run (3) a sharp change in slope is evident, the intersection of the two portions of the curve occurring at a pressure of 2·7 div. ≡ 0·054 mm.

In the initial stages of the illumination of the unoxidised sample, relatively little oxide will be available for conversion into the stable form; in the present case, therefore, where no induction periods are evident, extrapolation of the initial slope of curve (1) should cut the pressure axis at the final limit, or just above.

The results of such extrapolation, and of the subtraction of curve II from curve I in run (3), give the same limit of 0·054 mm., and this may be taken as the best value for the critical or 'dissociation' pressure at 25°C for pure chlorophyll.

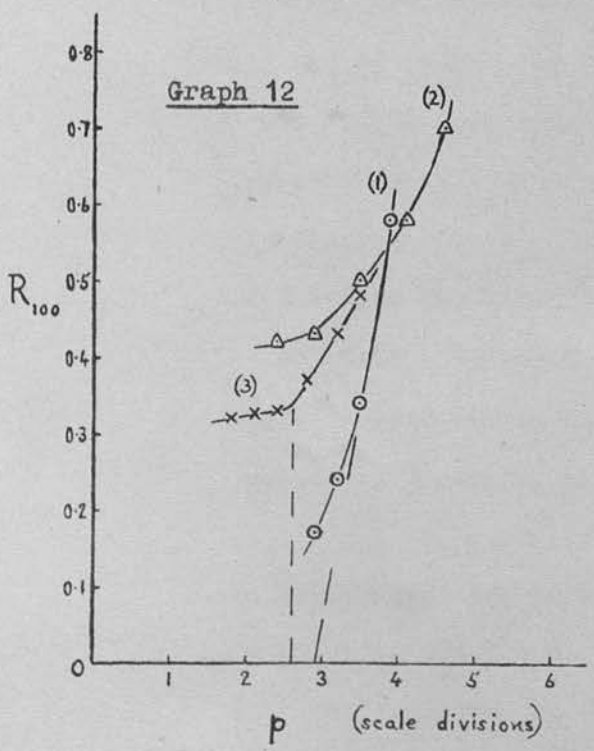
Spinach Chlorophyll - at 50°C
(on glass)

Graph 11



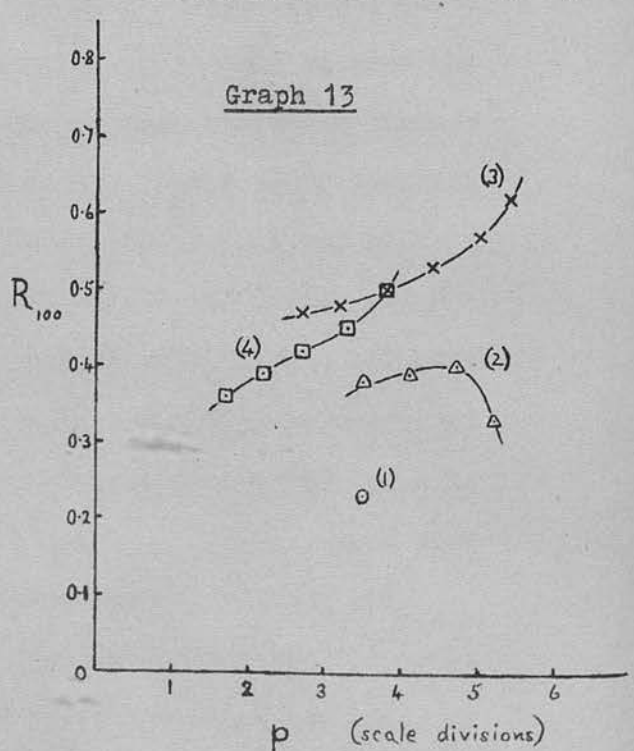
Spinach Chlorophyll - at 75°C
(on glass)

Graph 12



Spinach Chlorophyll - in presence
of P_2O_5 (on glass at 75°C)

Graph 13



The Effect of Temperature on the Low Pressure Photo-oxidation

In the investigation of the low pressure oxidation of pure chlorophyll at temperatures other than 25°C, a modification of the apparatus used in all the previous experiments was employed. Thus while the gauge was still maintained at 25°C, the reaction vessel was kept at a higher temperature, using the arrangement described on page 12. Films of spinach chlorophyll on powdered glass in the small vessel were used, and the results obtained compared with those of the previous experiment. The use of powdered glass as substrate instead of thallous bromide was considered advisable in view of the complications which seem to be involved with the latter under the conditions of these investigations.

The photo-oxidation at 50°C

Experimental. 1·73 mg. spinach chlorophyll
0·2 g. powdered glass (Winchester)
Gauge sensitivity = 0·020 mm./scale division
Small vessel at 50°C (± 0·1°).

Initial oxygen pressures in the illuminations of this film were (1) 9·2 div., (2) 6·3 div., (3) 8·2 div. and (4) 4·8 div.

	p	R_{100}		p	R_{100}		p	R_{100}		p	R_{100}
(1)	8·6	0·69		6·2	0·48		3·7	0·51		5·0	0·65
	8·2	0·57	(2)	5·8	0·65	(3)	7·7	0·82		4·3	0·61
	7·8	0·52		5·3	0·61		7·0	0·77	(4)	4·3	0·52
	7·3	0·50		4·8	0·57		6·3	0·75		3·5	0·52
	6·7	0·49		4·3	0·52		5·6	0·70		2·7	0·52

The general pattern of the curves obtained at 50°C (Graph 11) is similar to that at 25°C, but at somewhat higher pressures as

expected. The lowest pressure at which curves I and II intersect is 4·3 div. $\doteq$ 0·086 mm., in the combined rate curve (2) and (4). In consideration of the small slope of the second portion of this curve, which is comparable to that obtained in run (3) at 25°C, and also in consideration of the fact that below this pressure no curve I type was obtained, e.g. run (4), it would appear that this is the best value for the 'dissociation' pressure limit at 50°C for chlorophyll.

Extrapolation of the initial slope of curve (1) does not, however, cut the pressure axis near this critical limit as in the previous experiment. This may be due to a more rapid conversion of the reversible oxide into its stable form at the higher temperature, the section of the rate curve representing mainly the first process being too small to be appreciable. This would be accentuated by the fact that curve (1) in this experiment is not so far above the critical limit as in the corresponding case at 25°C.

It may be noted here also that the rates obtained at the pressures existing during (1) at 50°C, are considerably lower than might be expected from rates obtained subsequently at lower oxygen pressures; the previously observed anomaly of different rates for the same pressures in separate illuminations is thus again apparent.

The photo-oxidation at 75°C

Experimental. 1·73 mg. spinach chlorophyll
0·2 g. powdered glass (Winchester)
Gauge sensitivity = 0·041 mm./scale division
Small vessel at 75°C ($\pm$ 0·5°C)

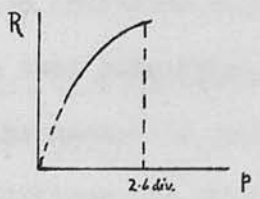
Initial oxygen pressures were (1) 4·4 div., (2) 5·1 div. and (3) 3·9 div.

	p	R_{100}		p	R_{100}		p	R_{100}	p	R_{100}
(1)	3·9	0·58	(2)	4·6	0·70		2·4	0·42	2·4	0·33
	3·5	0·34		4·1	0·58	(3)	3·5	0·48	2·1	0·32
	3·2	0·24		3·5	0·50		3·2	0·43	1·8	0·32
	2·9	0·17		2·9	0·43		2·8	0·37		

The rates obtained on first illuminating this film at 75°C decreased very rapidly, as shown on Graph 12; otherwise the general pattern of the curves is the same as at 25°C and 50°C. The intersection of the two portions of curve (3) occurs at a pressure of 2·6 div. ≡ 0·107 mm., the same limit being obtained by the subtraction of the curve II portion from curve I near the point of intersection. Here again extrapolation of the slope obtained on first illuminating the sample, cuts the pressure axis above this limit, while rates from subsequent illuminations in the same pressure region are again seen to be greater than those of run (1).

Repetition of this experiment at 75°C gave a similar series of curves, and confirmed the value for the 'dissociation' pressure of chlorophyll at this temperature as 0·107 mm.

At initial oxygen pressures just slightly greater than this limiting pressure, continuous rate curves were obtained of the form



This might correspond to the direct conversion of chlorophyll into its stable oxide.

Further experiments at low oxygen pressures

In considering the different rates observed under the conditions of these experiments, it is always to be emphasised that they refer to the decrease in pressure of the system and may not give the rates of oxidation directly. This fact may be examined from two points of view at this stage: (a) from a consideration of diffusion effects, and (b) with reference to the possibility mentioned previously (p. 5) that the pressure changes measured represent not simply an oxidation, but an oxygen uptake accompanied by the liberation of another resultant gas.

(a) The reaction taking place with thallous bromide as substrate at high oxygen pressures, has been shown to be a photosensitised reaction, in which the site of oxidation may be taken in the first instance as the surface of the thallous bromide crystal itself, such a site being separated from the gas phase by the thickness of the deposited chlorophyll. The observed rates using this substrate may therefore be conditioned mainly by the diffusion of oxygen through the film. This will depend on the film thickness and on the nature of the oxidation product. The latter will in general have a different oxygen 'permeability', giving greater or smaller rates of pressure decrease. On an inert support such as powdered glass, diffusion effects should not be so obvious since the predominant oxidation will be at the surface.

[15]

A change in the film thickness might be expected to magnify any such effect, and in this connection the experiment at 75°C was repeated using double the amount of powdered glass. Under otherwise identical conditions the rate curves obtained were of the same form as before (Graph 12), and a change in slope was shown

at approximately the same pressure as the critical limit (0·107 mm.) already found. In this case, however, the slope of the curve II portion was greater and consequently the exact point of intersection less readily determined. The more rapid fall off in rate observed in this particular stage of the reaction could conceivably be due to an increased screening of the chlorophyll by the glass. The difference however was small, and since no other change in the curves was evident it appears that the effect of diffusion, if rate-controlling here, must depend mainly on the nature of the oxidation product.

(b) The general rate curves obtained in the photo-oxidation of chlorophyll at low pressures are not reproducible, in the sense that in different runs different rates are obtained at the same pressures. Such non-reproducibility of results may be considered from two aspects: (1) reproducibility between different experimental preparations, and (2) reproducibility in any one experiment after stoppage of illumination, standing in oxygen, evacuation, etc. In (1) exact reproducibility might not be expected because of the unavoidable variation in the area of each film and the distribution of the chlorophyll on it, even when the same amount of powdered glass or of thallous bromide was used. In (2) at low pressures ($< 0{\cdot}5$ mm.) reproducibility should be obtained, but results show generally an increase in rate for the same pressure after evacuation. The difference cannot therefore be explained by allowing for a corresponding change in the chlorophyll concentration.

No evidence of this non-reproducibility has been reported at 100 mm. pressure, and the almost exact equivalence of chlorophyll and

oxygen (1 : 1) indicates that any retarding product of the reaction is not in large quantity. Such a product would be expected to be gaseous since evacuation seems to give an increased rate. Attempts to show the presence of any such compound in the gases left after illumination, proved negative. The quantities involved in these low pressure oxidations are, however, small, and negative tests not necessarily conclusive. It is also possible that some material, volatile slowly at low pressures, is introduced with the chlorophyll, such as a trace of adsorbed water.[44]

More definite evidence in this connection might be expected from carrying out the reaction in the presence of suitable adsorbents, and accordingly the experiment at 75°C was repeated in the presence of phosphoric oxide.

The photo-oxidation in presence of phosphoric oxide

Experimental. 1·73 mg. spinach chlorophyll
0·2 g. powdered glass (Winchester)
Gauge sensitivity = 0·041 mm./scale division
Small vessel with phosphoric oxide in side tube - at 75°C.

Initial oxygen pressures in the illuminations of this film were (1) 4·0 div., (2) 5·4 div., (3) 5·9 div. and (4) 4·2 div.

	p	R_{100}		p	R_{100}		p	R_{100}	p	R_{100}
(1)	3·5	0·23		3·5	0·38		3·8	0·50	3·3	0·45
(2)	5·2	0·33	(3)	5·4	0·62		3·2	0·48	2·7	0·42
	4·7	0·40		5·0	0·57		2·7	0·47	2·2	0·39
	4·1	0·39		4·4	0·53	(4)	3·8	0·50	1·7	0·36

The very slow decrease in pressure obtained on first illuminating here, and the acceleration period evident in run

(2) of Graph 13, are in contrast to the normal initial runs with no adsorbent present. Subsequent illuminations after evacuation, however, show an increase in the rate for the same pressure as before. It is difficult to see how the decreased initial rates can be connected with the presence of the phosphoric oxide, since the removal of any retarding product by it would be expected to result instead in an increase in rate, unless water is a necessary catalyst, initially present in the film and here predominantly removed initially, and produced by the oxidation later, being then incompletely removed under the conditions.

No marked change in slope is apparent in this experiment in the region of the 'dissociation pressure' for the temperature concerned, the rate curve (4) being almost continuous. It is difficult to interpret such results on the basis of the reversible oxide hypothesis.

The effect of adsorbents on the general photo-oxidation of chlorophyll is examined under different conditions later in this work.

While all previous observations have been explained on the basis of reversibility, no evidence of an actual pressure increase was obtained at any time, either (a) on shutting off the light or (b) on reducing the pressure after an oxidation. Such an increase might be expected from the decomposition of the reversible oxide, and would presumably represent a unimolecular reaction. Previous
45
results have shown that 'reversal' at low oxygen pressures is very rapid, an increase in rate being apparent at 100 mm. pressure

after only 5 minutes evacuation. In (b) above, the time involved in reducing the pressure may be too long for the observation of any increase, but the results of (a), where only the time of shutting off the light is involved, indicate that the amount of reversible oxide at these low pressures is very small.

The reaction scheme developed previously (see p. 4), leading to the kinetic formula, has been based on evacuation results, etc., but it is a general possibility that the system is a photostationary state, with an equilibrium oxygen pressure depending on the light intensity. No change was observed, however, in the system on altering the light intensity at the critical pressure limit already established for a given temperature, and consequently such a possibility may be precluded.

Photo-oxidations at High Oxygen Pressures - Normal Oxidations

A number of experiments were carried out under conditions similar to those employed by previous investigators[13], with a view to the further elucidation of the nature of the changes occurring after the illumination of chlorophyll in oxygen. The pressures used here were far in excess of the 'dissociation' pressures measured in the previous section, and since the amount of pigment present during any one experiment was fully oxidised when a pressure decrease of less than 2 mm. had occurred, the pressure throughout such oxidations could be taken as constant. The results obtained were therefore best interpreted by plotting reaction rate against pressure decrease. By this method a direct measure of the molecular ratio of total gas pressure decrease to oxidisable material was obtained.

The copper-containing chlorophyll sample was used in most of these investigations[13c], since previous experiment indicated that it oxidised in an essentially similar manner to the pure chlorophyll obtained from spinach leaves, and is at the same time easier to handle, having been found to keep intact for longer periods in solution. Oxidations were examined both on thallous bromide and on powdered glass films, using 'white' light illumination and a temperature of 25°C throughout. For comparison, some experiments were first carried out on the bonemeal-separated copper-free chlorophyll sample.

Copper-free Chlorophyll (on thallous bromide)

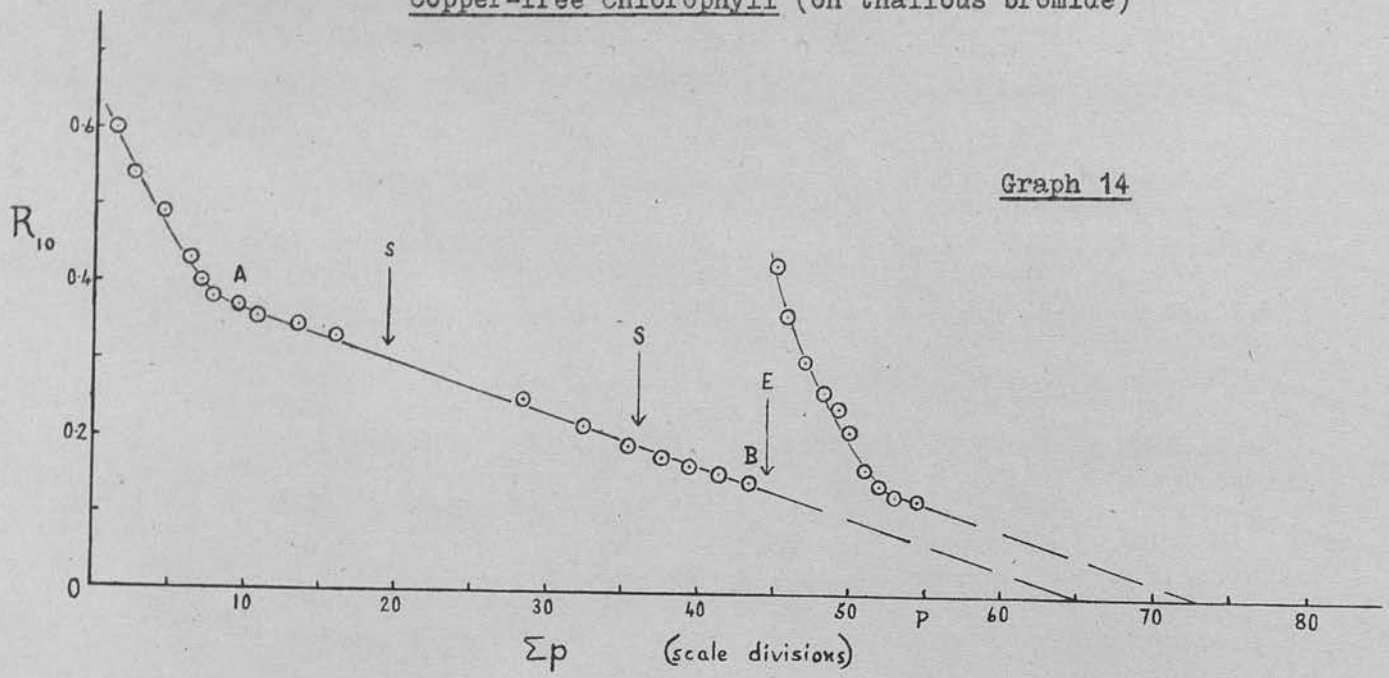
Experimental. 2·65 mg. chlorophyll (copper-free sample)

0·3 g. thallous bromide

Gauge sensitivity = 0·020 mm./scale division

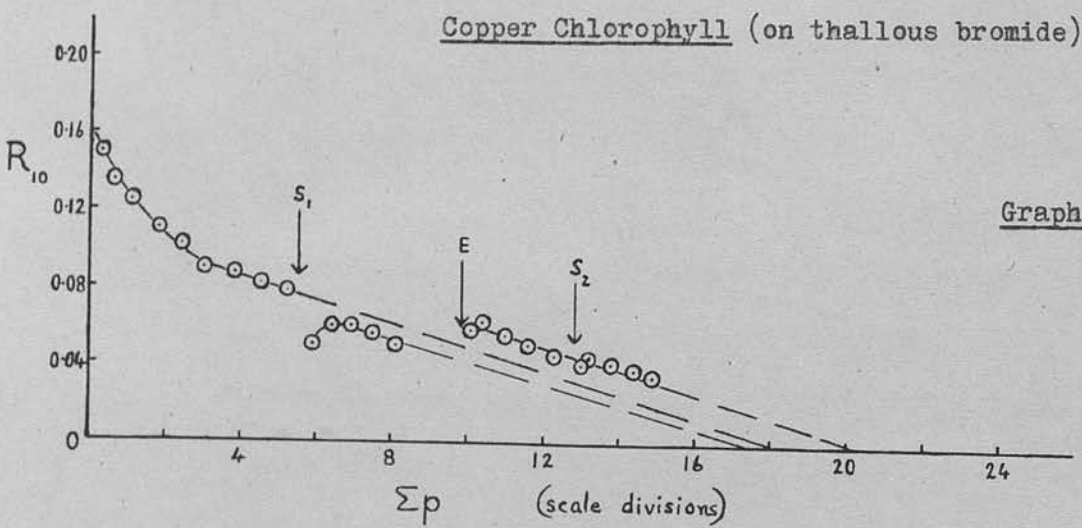
Copper-free Chlorophyll (on thallous bromide)

Graph 14



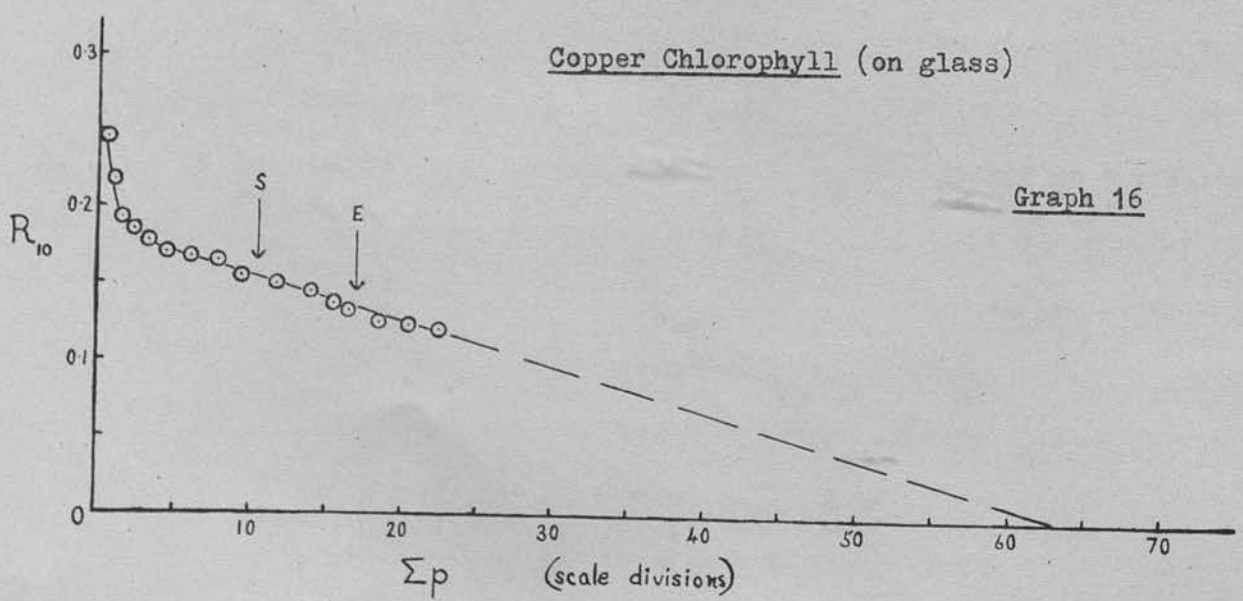
Copper Chlorophyll (on thallous bromide)

Graph 15



Copper Chlorophyll (on glass)

Graph 16



Volume of reaction space = 79·45 ml.

Oxygen was added to a pressure of 100 mm., and the film illuminated when steady conditions had been reached. From the pressure-time curve obtained the following rates were found.

Σp	R_{10}	Σp	R_{10}	Σp	R_{10}	Σp	R_{10}
1·3	0·60	10·8	0·355	39·5	0·165	49·3	0·240
2·4	0·54	13·6	0·345	41·5	0·155	50·1	0·210
4·4	0·49	16·0	0·330	43·5	0·145	51·0	0·160
6·2	0·43	28·5	0·250	45·0	0·425	52·0	0·140
7·0	0·40	32·5	0·215	45·8	0·360	53·0	0·125
7·8	0·38	35·5	0·190	47·0	0·300	54·5	0·120
9·4	0·37	37·7	0·175	48·3	0·260		

where Σp represents the total pressure decrease at any point expressed in scale divisions, and R_{10} represents the rate at the same point expressed in scale divisions per 10 minutes.

At the positions S on Graph 14, illumination was discontinued and the film allowed to stand in the dark at 100 mm. pressure for several hours before recommencing illumination. At E the light was again cut off and the system evacuated; after an interval in vacuo, oxygen was re-admitted to 100 mm. pressure and illumination recommenced.

The total pressure decrease corresponding to zero rate was found by extrapolation of the linear portion AB of the rate curve. For the weight of reactant taken here, 2·65 mg., this extrapolated value OP is 65·0 scale divisions $\equiv$ 65·0 × 0·020 mm. Knowing the volume of the system and the temperature, it is possible to calculate the molecular ratio of gas pressure decrease to oxidisable material. For this calculation the molecular weight

of the material is required, and since in all these experiments the chlorophyll examined consists of a mixture of the a and b components, of molecular weights 893 and 907, this may be taken without much error as 900.

The ratio of the number of gas molecules decrease to the number of chlorophyll molecules used is then

$$\frac{79{\cdot}45 \times 65{\cdot}0 \times 0{\cdot}020 \times 273 \times 1000 \times 900}{22400 \times 760 \times 298 \times 2{\cdot}65} = 1{\cdot}89$$

The accuracy of determinations such as this depends mainly on the calibration of the gauge sensitivity, in which the error was considered to be about 3%. The value found here is in good agreement with that reported by previous workers for the same sample of chlorophyll.[13b,c] Repetition of this experiment at an oxygen pressure of 20 mm. gave a molecular ratio of 2·06, and at 10 mm. pressure a ratio of 1·82, showing that the result obtained is substantially independent of the pressure at which the reaction is carried out. On the assumption that the pressure decreases observed are due entirely to the uptake of oxygen, the ratio obtained corresponds to the uptake of approximately two molecules of oxygen by each molecule of chlorophyll on illumination.

On the same basis, the increase in rate observed after evacuation, giving a rate curve equal in slope to that of the original and an increased extrapolated value, indicates the oxidation to have been reversed to a certain extent.

As seen from Graph 14, dark periods in oxygen during which the pressure was unchanged have no apparent effect on the rate of pressure decrease. The same was found in the oxidation at 10 mm. pressure, where rates after such intervals were found to lie on

the extrapolation of the original curve. The reversible oxide appears therefore to be stable at these pressures. This observation may be compared with that of Lonie, who found an appreciable increase in rate after dark periods at pressures as high as 50 mm.[46]; in these experiments, however, the oxygen pressure during illumination was 100 mm., being reduced subsequently to 50 mm., the increased rate being observed on re-illumination at 100 mm. pressure. The effect is reported only for the copper chlorophyll sample too, the oxidation of which has always been found to 'reverse' to a greater extent than that of the copper-free sample.[13b,c]

As mentioned earlier (p. 41), the molecular ratio of approximately two obtained with the copper-free chlorophyll has been taken as indicating its partial degradation to pheophytin, and accordingly its oxidation was not investigated further.

Copper Chlorophyll (on thallous bromide)

The general form of the rate curves obtained on illuminating the copper-containing sample of chlorophyll adsorbed on thallous bromide, at different overall oxygen pressures, was similar to that observed with the copper-free sample. At 100 mm. pressure, values found for the molecular ratio of pressure decrease to chlorophyll, using different amounts of chlorophyll, were 1·04 and 1·01. An increase in rate was again observed after evacuation.

The results obtained in an experiment at 20 mm. pressure are quoted below.

Experimental. 3·42 mg. chlorophyll (copper-containing sample)
0·3 g. thallous bromide
Gauge sensitivity = 0·053 mm./scale division

Volume of reaction space = 75·8 ml.

The rates obtained from the illumination of this film at 20 mm. pressure were as follows -

Σp	R_{10}	Σp	R_{10}	Σp	R_{10}	Σp	R_{10}
0·3	0·150	3·8	0·087	7·5	0·056	12·3	0·045
0·6	0·135	4·5	0·082	8·1	0·050	13·0	0·040
1·1	0·125	5·2	0·078	10·1	0·058	13·2	0·045
1·8	0·110	5·9	0·050	10·4	0·062	13·8	0·041
2·4	0·102	6·4	0·060	11·0	0·055	14·4	0·038
3·0	0·090	6·9	0·060	11·6	0·050	14·9	0·035

Extrapolated Σp = 18·0 divisions. Molecular ratio = 1·02.

It is evident from this result, that the molecular ratio is independent of the pressure at which the oxidation is carried out. The value of unity observed here is in agreement with that shown previously to hold for pure chlorophyll.[13c]

From Graph 15 it is seen that rates after the first dark period in oxygen at 20 mm. pressure, at the position marked S_1, are lower than might have been expected from the extrapolation of the original curve, and apparently tend to a slightly reduced extrapolated value. The curve obtained after evacuation (at E) followed by re-illumination at the same pressure, is approximately parallel to and higher than the extrapolation of the original. This increase in rate may be due wholly to the 'reversal' effect found normally on evacuation, or in part also to the removal of the retarding effect observed at S_1. No further retardation was observed after a period of standing in oxygen at a later stage in the reaction (at S_2), the final extrapolated value being greater than that of the original curve.

In runs at 100 mm. oxygen a similar retardation in rate was observed after dark periods during which the pressure was unchanged. In all cases this decrease in rate was found to occur when the observed pressure decrease was less than half the total extrapolated value, similar treatment at later stages in the reaction resulting in no further apparent rate decrease. The actual pressure of the system during such an interval was found to remain unaltered.

The only mention of an effect such as this in previous work of a similar nature, is the retardation in rate reported in experiments with a copper chlorophyll-carotenoid-phosphoric acid film after intervals of standing in oxygen.[47] In this case the rates were found to rejoin the original curve after 'induction' periods. The effect was considered to be due to the pheophytin produced by the acid and not to the carotene present. In the present experiments no acid is incorporated in the film and the molecular ratio is unity; the retardation appears therefore to be connected with the chlorophyll system itself.

The Photo-oxidation on Glass

A similar examination was made of the copper chlorophyll sample when deposited on powdered glass. In runs at 100 mm. oxygen pressure, values found for the ratio of gas molecules pressure decrease to chlorophyll molecules present, using different amounts of pigment adsorbed on the same amount of glass, were 1·02, 0·95, 0·90 and 1·25. The average value of 1·03 is sufficiently close to unity to be taken as such. Thus the molar ratio for chlorophyll is the same when measured on an inert substrate such as powdered glass as on thallous bromide.

The form of the rate curves obtained was also similar. In two parallel experiments, the one with 0·3 g. thallous bromide as substrate and the other with an equal weight of powdered glass (Winchester), the rates on glass were found to be approximately two thirds of those on thallous bromide, at corresponding percentages of the total pressure decrease, the molar ratio in the latter case being 1·04 and in the former 1·02. A more exact comparison of rates is not possible, owing to the unavoidable variations between different experimental preparations, all other conditions being equal.

The results obtained in an experiment at 20 mm. oxygen pressure, similar to the one quoted above for chlorophyll on thallous bromide, were as follows -

Experimental. 3·42 mg. chlorophyll (copper-containing sample)
0·2 g. powdered glass (Winchester)
Gauge sensitivity = 0·053 mm./scale division
Volume of reaction space = 23·5 ml.
Oxygen pressure = 20 mm.

Σp	R_{10}	Σp	R_{10}	Σp	R_{10}	Σp	R_{10}
0·5	0·245	3·2	0·178	9·3	0·155	16·5	0·132
1·0	0·218	4·3	0·170	11·7	0·150	18·5	0·125
1·4	0·192	6·0	0·168	14·0	0·145	20·5	0·123
2·2	0·185	7·7	0·165	15·5	0·138	22·5	0·120

Extrapolated Σp = 63·0 divisions. Molecular ratio = 1·11.

The molecular ratio is again seen to be independent of the pressure at which the reaction is carried out.

The behaviour of the system on re-illumination at the same pressure, after dark periods in oxygen or in vacuo, differs from that observed in experiments with thallous bromide as substrate. Thus no marked retardation in rate is evident after 13 hours in the dark at S (Graph 16). A small decrease in the pressure of the system (approximately 0·6 div.) was observed to have occurred during this interval; this may indicate the dark adsorption of some gaseous reaction product, the effect being too small to show as an alteration in the rate. The absence of any marked increase in rate after evacuation (at E on Graph 16) is also in contrast to the effect normally observed with chlorophyll on thallous bromide. From this it appears that either the oxidation is reversed to a smaller extent on glass, or the change from reversible oxide to the final stable form is more rapid.

Such differences in the behaviour on the two substrates under the present conditions, are similar to those observed in the work at low oxygen pressures.

The mechanism proposed (see p. 4) to explain the oxidation of chlorophyll under the conditions of these experiments included the reactions

$$\text{Chl} + O_2 \overset{h\nu}{\rightleftharpoons} \underset{\text{(reversible)}}{\text{Chl.}O_2} \longrightarrow \underset{\text{(stable)}}{\text{Chl.}O_2}$$

in which the change from reversible to stable oxide may be either thermal or photochemical, since the possibility exists that the primary reversible product is still coloured and can absorb the incident radiations.

To investigate the nature of this possible change, an experiment was carried out in which two films of copper chlorophyll on thallous bromide were illuminated at 100 mm. oxygen pressure until the same pressure decrease, approximately one third of the total, had been observed for both. One film was then subjected to evacuation and after several hours was re-illuminated at 100 mm. pressure; the other was allowed to stand in oxygen in the dark for approximately 60 hours, then evacuated for a similar length of time as the first film, and re-illuminated at 100 mm. The normal increase in rate was observed in the first instance after evacuation, but in the second, rates were found to lie slightly below the extrapolation of the original curve. This seemed to indicate that the change from reversible to irreversible product had occurred during the dark interval preceding evacuation, the mechanism therefore being thermal and complete in this instance in 60 hours.

Repetition of this experiment with another solution of chlorophyll, obtained from the same copper-stabilised preparation, did not however confirm this conclusion. Thus an appreciable increase in rate was observed after an evacuation preceded by a dark interval of $5\frac{1}{2}$ days at 100 mm. oxygen, where the pressure decrease observed had been approximately 40% of the extrapolated value.

In considering these results and the discrepancy between them, certain factors must be taken into account. The molar gas decrease to pigment ratio (1·6) obtained in the repeat experiment was nearer that given by the copper-free chlorophyll sample than the unit ratio connected with pure chlorophyll. This suggests

that partial degradation of the copper chlorophyll preparation had occurred, similar to that observed with the non-stabilised sample, the solution used in this experiment having been prepared at a later date than that used in the first experiment. Such degradation has been attributed to the partial conversion of the chlorophyll to pheophytin, though in this case no corresponding change in the colour and absorption spectrum of the solution was observed (cf. p. 41).

A similar examination was made of pure pheophytin on thallous bromide. This compound has been shown to give a molar ratio of three and to exhibit a small 'reversibility' on evacuation.[13c] It was found that evacuation following a dark interval in oxygen, after a pressure decrease equivalent to almost one molecule of gas had been observed, resulted in no obvious change in rate, whereas at a later stage in the oxidation an increase was obtained after such treatment.

Since with pheophytin more than one position in the molecule is being oxidised, it appears from these observations that the mechanism of conversion from reversible to stable forms is not the same for all. The positions in question are presumably the one normally attacked in the chlorophyll molecule, and the NH groups made available to oxidation by the removal of the central magnesium atom. The results of these experiments on chlorophyll and pheophytin appear to indicate that, on the reversible oxide theory, the conversion of the first (chlorophyll) oxide to its stable form is thermal, while that of the NH positions is photochemical. The increase in rate observed after evacuation in the later stages of the pheophytin oxidation, must then have been due

to the reversion of the oxidation of these NH positions, and that occurring in the 'repeat' experiment with copper chlorophyll to the presence of a small amount of pheophytin.

The concept of reversibility developed previously (see p. 4) is based mainly on observations of the behaviour of such solid pigment systems on evacuation or reduction of pressure. No direct evidence has been obtained of an actual increase in pressure under these conditions; thus after illuminating chlorophyll at 100 mm. oxygen, reducing the pressure by the oil pump, and then observing the gauge pointer, no change in pressure was evident. A similar result was obtained in work at low oxygen pressures (p. 63). While it was possible that at low pressures the amount of reversible oxide was too small to show an appreciable pressure increase on evacuation, at 100 mm. oxygen the amounts involved should be greater.[45] 'Reversal' at low pressures has been shown to be very rapid, however, and it was possible that the time involved in reducing the pressure of the system by oil pump evacuation was greater than that required for the reversible oxide to undergo dissociation.

A considerable reduction in this time was achieved by the rapid expansion of the gas present after the oxidation into a large evacuated bulb attached to the apparatus. The maximum rate at which this operation could be performed was conditioned mainly by the relative difference in volume between the gauge sickle and its jacket, the time involved in the expansion depending on this and on the volume of the subsidiary bulb used.

In a blank experiment in which 100 mm. dry air was expanded rapidly into a subsidiary 2 litre bulb, and the gauge pointer position then observed at intervals, it was found that the pointer rose slightly. This was probably due to a cooling of the gas on expansion, followed by a change in the pointer position as the temperature returned to its steady value. The time interval from beginning the expansion, indicated by the first movement of the gauge pointer, to closing the gauge taps and noting the pointer position, was approximately 1 minute, the final pressure obtained being one third of the original. The actual pressure change observed (0·3 scale divisions with a gauge of sensitivity 0·053 mm. per division) was small, and it was considered possible therefore that the same procedure, if adopted after the oxidation of a film of chlorophyll, would show any increase in pressure resulting from a reversion of such oxidation as a marked increase in this effect.

In practice this was not found to be so. Thus with films of copper chlorophyll on thallous bromide, reduction of the pressure to one third by rapid expansion after illumination at 100 mm. oxygen, gave pressure increases approximately equal in magnitude to those observed when only the expansion of the gas was involved. Comparison of the rates at which the pressure increased under different conditions was uncertain, since the changes involved were small and their measurement correspondingly inaccurate. For the same reason, no definite conclusion may be drawn as to the nature of the effect, from the fact that neither the unimolecular nor the bimolecular rate equations were found to fit the curves obtained by plotting the observed pressure increases against time. The figures for the corresponding unimolecular and

bimolecular rate constants obtained from one experiment were

$k_1 = 5{\cdot}07 \times 10^{-3},\ 5{\cdot}11 \times 10^{-3},\ 4{\cdot}58 \times 10^{-3},\ 3{\cdot}99 \times 10^{-3},\ 2{\cdot}84 \times 10^{-3}$

$k_2 = \quad 5206, \qquad 5260, \qquad 4728, \qquad 4134, \qquad 2932.$

The possibility remains, however, that reversion of the oxidation by pressure reduction has resulted in a pressure increase greater than that actually observed, this increase having occurred during the time of expansion. In one such oxidation and expansion, when the total increase in pressure observed after a period of 13 hours was 0·5 divisions, the rates obtained on re-illumination at 100 mm. pressure were found to return to the extrapolation of the original curve, no 'reversion' being evident. This seems to point to a temperature effect as the cause of the increase observed on expansion, but it is possible that the measurement of rates is not sufficiently accurate to show a reversal effect of this magnitude.

The extent of the 'reversion' with chlorophyll has been shown to depend on the pressure to which the system is reduced, becoming appreciable at pressures in the region of 50 mm. and increasing to a maximum on evacuation.[46] Reduction of the oxidation pressure to values lower than 33 mm. should, therefore, result in a greater increase in pressure on expansion. Such could be achieved by the use of a larger subsidiary bulb, and in the present experiments seemed desirable. The time involved in expansion under these conditions, however, was greater and prevented the observation of the early stages of any reverse process. A similar disadvantage would attend the use of a reaction vessel of smaller volume, or a gauge of greater sensitivity, cancelling out the advantage of magnified changes in pressure.

The Effect of Temperature on the Rate of Photo-oxidation

The temperature coefficient of photosynthesis in plants is appreciable in contrast to what would be expected of a purely photochemical reaction. This has been attributed to accompanying dark reactions[48], the exact number and nature of which is uncertain, but included in which, according to Willstätter and Stoll[49], is the splitting off of oxygen from peroxides. Many investigations, starting with those of Blackman and Matthaei[50], have shown a value of 2 for the overall photosynthetic coefficient; a certain dependence on the light intensity and the carbon dioxide concentration has also been demonstrated[51].

In the present experiments there would appear to be three possible rates capable of temperature variation:

(a) the rate of labile peroxide dissociation (see pp. 57-59)

(b) the rate of pressure change at low oxygen pressures (< 0·5 mm.)

(c) the rate of pressure change at high oxygen pressures (e.g. 100 mm.)

In both (b) and (c), because of the difficulties of reproducible film formation, it is necessary to measure relative rates at two temperatures in the one illumination. For the low pressure oxidation it would seem better also, that such measurements be recorded for a fresh, i.e. unoxidised, sample of chlorophyll, since with such the possible conversion of reversible oxide to stable will be relatively small, and this may have a temperature coefficient of its own.

(b) At low oxygen pressures

A film of spinach chlorophyll on powdered glass was illuminated at an initial oxygen pressure of about 0·4 mm., and at a

temperature of 75°C (± 0·5°), and measurements of the pressure made at intervals over a suitable period, until steady state conditions free from induction periods were obtained. The temperature of the reaction vessel was then changed to 25°C (± 0·5°), without interrupting the illumination, and after a suitable interval to 75°C (± 0·5°) once more. By applying suitable factors, all pressure readings were converted to a common temperature (273°A), and from the pressure-time curves thus obtained, rates were measured. Comparison of the rates at 75°C, before and after the interval at 25°C, with those obtained during that interval, at the pressures concerned, showed very little change in rate to have occurred with temperature. The actual ratio of the rates $\frac{R_{75^\circ}}{R_{25^\circ}}$ was $\frac{0{\cdot}47}{0{\cdot}54} = 0{\cdot}87$, indicating a zero or slightly negative temperature effect under these conditions.

Repetition of this experiment at an initial temperature of 25°C, followed by a period at 75°C, and finally a return to 25°C, again showed the influence of temperature on the rate to be very small. The ratio observed in this case was equal to 0·95 $\left(\frac{R_{75^\circ}}{R_{25^\circ}} = \frac{0{\cdot}62}{0{\cdot}65}\right)$.

Since the differences observed between the rates at the two temperatures were considered to be within the limits of experimental error, no exact significance may be attached to the actual ratios obtained. In general, however, it may be concluded that, for the oxygen pressures concerned and for the temperature range 25-75°C, the rate-controlling reaction is not appreciably affected by temperature and may therefore be taken to be mainly photochemical.

(c) At high oxygen pressures

Illumination of a film of spinach chlorophyll on glass at a temperature of 25°C and an oxygen pressure of 100 mm., followed by an alteration in the reaction temperature to 75°C, without interruption of the illumination, showed that in this case the reaction rate was considerably influenced by temperature. By extrapolation of the linear portions of the rate curves obtained at the two temperatures, it was possible to compare rates for the same point in the reaction, namely that point at which the temperature was altered, and a value for the ratio $\frac{R_{75^\circ}}{R_{25^\circ}}$ of 2·5 was obtained. The possibility that the increased rates observed were due to a reversion of the oxidation at the higher temperature used, was eliminated by an experiment in which an oxidation was carried out at 75°C, and interrupted by a dark period of several hours at that temperature, no change in the rate being evident after such treatment. Since, however, the molar ratio of pressure decrease to oxidisable material (2·9) obtained in this experiment, was nearer that given by pheophytin than the unit ratio associated with pure chlorophyll (cf. p. 41), it seemed possible that at the high temperature involved, a certain amount of degradation of the chlorophyll had occurred. The appearance of the film after illumination in both experiments confirmed this, the clear green colour of the unbleached chlorophyll having changed to the olive green characteristic of pheophytin. In view of this the ratio of the rates at 75°C and 25°C observed under these conditions, cannot be considered as representing the temperature effect for pure chlorophyll, and in consideration of this fact the investigation was not pursued any further.

The Determination of Peroxidic Oxygen in Photo-oxidised Chlorophyll

The method used to determine the amount of peroxidic oxygen present in films of chlorophyll after illumination in oxygen, depends on the estimation of the colour developed on the addition of an acid solution of ferrous thiocyanate in methanol. In investigating the relationship between this amount and the decrease in pressure observed during the illumination, films of the copper-containing chlorophyll were used, since the pressure changes observed with this sample have been shown to be equivalent to those occurring with pure spinach chlorophyll.[13c]

Copper Chlorophyll

In a preliminary experiment, in which a film of copper chlorophyll on powdered glass was illuminated at 100 mm. oxygen, the pressure decrease noted, and then the film removed and estimated for peroxide as described on page 28, the amount of peroxidic oxygen found was approximately equivalent to the pressure decrease observed. A similar film kept in the dark while the first one was illuminated, did not give any appreciable peroxide reaction on the addition of the ferrous thiocyanate reagent. From this it may be concluded that the colour developed in the first instance was due entirely to the peroxide formed on illumination, and not to any change in the chlorophyll brought about by conditions in the solid state.

A similar preliminary estimation of the amount of peroxide formed on the illumination of copper chlorophyll adsorbed on thallous bromide, showed that only about one tenth of the pressure

decrease observed was detectable as peroxide. The marked difference between this result and the previous one appeared to be due to a stronger adsorption of the oxidation products on the thallous bromide crystals, and in view of this, films on powdered glass were used in the more detailed investigation.

Experimental. Films of 2·06 mg. chlorophyll (copper-containing sample), deposited on 0·3 g. powdered glass (Winchester), in spherical reaction vessels, were illuminated at 100 mm. oxygen pressure until a pressure decrease of more than half the total extrapolated amount had been observed, the illumination in each case being continuous.

In the first three experiments, the film was extracted with methanol immediately after illumination, and the amount of peroxidic oxygen present estimated as described on page 28, the measurement being completed within an hour of ceasing illumination.

In experiments 5 and 6, the film was evacuated after illumination and allowed to stand in vacuo for some time (1 day and 14 days respectively), before being extracted and estimated in the same way.

Experiment 4 represents an attempt to reduce the time interval between the first extraction of the oxide and its estimation with ferrous thiocyanate reagent. In this case the film was extracted directly with the reagent after illumination, and the colour developed estimated as before. The time involved was approximately 25 minutes.

The following table summarises the results obtained in these six experiments. The actual pressure decrease observed with each film is expressed as μg of molecular oxygen, while the amount of

peroxidic oxygen, found by comparison with the hydrogen peroxide calibration curve, is given as a percentage of this. The extent of the oxidation in each case is represented by column 3, in which the percentage of the total pressure decrease calculated from the $R_{10}/\Sigma p$ curve is given. The unit molecular ratio was observed in each experiment.

No. of expt.	Pressure decrease (μg. O_2)	Percentage total pressure decrease	Percentage pressure decrease detected as peroxidic oxygen	
			(a)	(b)
1	41	56	102	96
2	43	68	81	77
3	33	45	79	70
4	41	56	90	85
5	37	54	92	80
6	35	48	83	77
7	55	73	27	23

For convenience the results of a similar experiment, in which a film was taken of chlorophyll deposited on 0·3 g. thallous bromide instead of on powdered glass (experiment 7), are included in this table.

Two values for the percentage of peroxidic oxygen are quoted in each experiment: those in column (a) were found on the assumption that any absorption due to unoxidised chlorophyll remaining after illumination, is negligible in comparison to the absorption of the ferric thiocyanate complex, while column (b) represents values corrected for this effect. The latter were found as described on page 28, the amount of unoxidised chlorophyll being calculated in each case from the rate curve, on the assumption that no reversion of the oxidation occurred on

dissolution in methanol. Since the partial pressure of oxygen in this solvent (64 mm.)[52] is less than atmospheric, this may not be strictly true, and such percentages must consequently, from the point of view of the reversible oxidation theory, be considered as minimum values.

From the results of experiments 1, 2 and 3 it is seen that exact reproducibility is not obtainable, a variation of as much as 26% being found even when the procedure adopted in each case was as nearly identical as possible. The average values for the percentages of the observed pressure decreases detected as peroxide, from these three experiments are (a) 87 and (b) 81, and these may be considered as average maximum and minimum values. The corresponding averages obtained by comparison with the decalin hydroperoxide calibration curve (see p. 29) - (a) 87 and (b) 77 - agree reasonably closely with these values.

In view of the unstable nature of hydroperoxides in general,[53] one might not expect to be able to detect as peroxide all the oxygen taken up on illumination. For the same reason the peroxide percentages obtained in experiment 4 should be greater than the average values of the previous experiments, the time involved in this determination being less. The actual differences observed, however, are not outside experimental error.

The results obtained when peroxide estimation was preceded by evacuation, do not appear appreciably different from those obtained immediately after illumination; thus the average values from experiments 5 and 6 are (a) 87·5 and (b) 78·5. If any marked reversion of the oxidation had occurred on evacuation, it might have been expected to show as a decrease in the amount of peroxide

detected. In this connection it may be noted, however, that the extent of the reversibility of chlorophyll photo-oxidation on glass has been shown to be comparatively small (p.72), and in view of the irreproducibility evident in the present results, may not be detectable. From the result of experiment 6 in particular, it is evident that no appreciable decomposition of the oxidation product takes place during a prolonged period in the solid state in vacuo.

In experiment 7, in which thallous bromide was used as film substrate instead of powdered glass, the amount of peroxidic oxygen detected was very much less than in any of the previous estimations. Since the crystals of thallous bromide after repeated extraction with methanol were seen to be slightly green, the low value obtained was considered to be due, in part at least, to the incomplete removal of the oxidation products, which appeared, therefore, to be more strongly adsorbed on thallous bromide than on glass. The low result may be due also partly to a more appreciable reversion of the oxidation on thallous bromide on dissolution in methanol, compared to that on glass. In view of the adsorption factor apparently involved in this experiment, no direct comparison between the oxidations using the different substrates is possible by this method. Similarly any investigation of the extent of reversion on evacuation with thallous bromide films is impracticable.

It is impossible to assign any precise order of accuracy to the method used in estimating peroxidic oxygen in these oxidised chlorophyll films, since no absolute means of checking is available. In summarising the results obtained it may be said that, of the pressure decreases observed on illumination in

oxygen of copper chlorophyll deposited on glass, values of peroxide equivalent to approximately 80% have been observed. If these pressure decreases refer simply to the oxidation of the chlorophyll, then it may be concluded that almost all the oxygen taken up on illumination combines in the peroxidic form. Moreover, since it was always found in estimating these peroxides that the colour with the ferrous thiocyanate reagent was developed immediately, the compound formed on illumination may be taken to be a hydroperoxide.[54]

Spinach Chlorophyll

A similar estimation was made of the amount of peroxidic oxygen present in an oxidised film of spinach chlorophyll.

Experimental. A film of 0·5 mg. spinach chlorophyll on 0·3 g. powdered glass (Winchester), in the spherical vessel, was illuminated until a pressure decrease of more than half the total extrapolated value was obtained. It was then extracted immediately with methanol and the peroxidic oxygen content of the extract found as before.

The pressure decrease observed was equivalent to 25 μg. molecular oxygen, and the amount of peroxidic oxygen found was (a) 87% of this and (b) 78%.

The close agreement of this result with the average value found for copper chlorophyll under similar conditions, is a further indication that the reaction taking place on illumination in oxygen is the same for both samples.

Copper-free Chlorophyll

A film of the copper-free chlorophyll sample on glass, when illuminated in oxygen and then estimated in the same way, gave

a peroxide value equivalent to a percentage pressure decrease of the same magnitude [(a) 114%, (b) 99%].

Pheophytin

After illumination in oxygen, a film of pheophytin on glass was extracted with methanol and the volume of the extract made up to 25 ml. Known volumes of this solution were estimated for peroxide after various intervals, and from the values found the total peroxide content of the extract was calculated. An average value of separate determinations is given in each case below.

Pressure decrease (μg. O_2)	Percentage total pressure decrease	Time of measurement	Percentage pressure decrease detected as peroxidic oxygen
226	68	1 hour	126
		3 days	101
		7 days	84
		28 days	78

The peroxidic oxygen percentages quoted here are the maximum, or (a) values; the corresponding (b) value for a time of measurement of 1 hour was 89%. The large difference between this and the value of 126% suggests that a certain amount of the pheophytin had been oxidised before illumination.

The results show the decrease in concentration of peroxide with time, and indicate that this occurs fairly rapidly at first and then becomes almost steady. The (b) value obtained shows that with pheophytin also, where three molecules of oxygen are taken up per molecule of pigment, instead of one as with chlorophyll, almost all the oxygen taken up on illumination combines in the hydroperoxidic form.

Phytol

One of the esterifying alcohols of the chlorophyll molecule[5], phytol, contains an olefinic bond, and it is a general possibility that in chlorophyll attack by molecular oxygen on illumination occurs at this position[55], or at the adjacent α methylenic group[56]. Some evidence in support of this has been found recently; thus it has been shown that illumination in oxygen of a film of the alcohol deposited on thallous bromide, gives a pressure decrease equivalent to a unit molecular ratio[57]. In view of this it was of interest to examine the pure alcohol in a way similar to that used above with chlorophyll.

Experimental. Films of 2 ml. of a 1·018 g./litre solution of pure phytol (B.D.H.) in ether on 0·3 g. powdered glass (Winchester) in spherical reaction vessels, were illuminated at 100 mm. oxygen pressure until an appreciable pressure decrease had been observed.

In experiment 1 the film was extracted with methanol after illumination and known volumes of the extract estimated for peroxidic oxygen as before, immediately and then after an interval of 48 hours.

In experiment 2 the film was extracted directly with ferrous thiocyanate reagent and estimated.

Since phytol is colourless, its rate of oxidation on glass was very slow, and long periods of illumination were required to produce appreciable pressure changes. In calculating the extent of oxidation in each experiment, therefore, the unit molecular ratio was assumed to hold and from this the total pressure decrease was estimated. With phytol there was no necessity to correct for absorption by any unoxidised material remaining after illumination.

No. of expt.	Pressure decrease (μg. O_2)	Percentage total pressure decrease	Time of measurement	Percentage pressure decrease detected as peroxidic oxygen
1	61	28	1 hour	53
			48 hours	53
2	36	17	20 minutes	28

Comparison of the estimated peroxide values obtained here with those of the previous experiments, indicates that if all the oxygen taken up on illumination, as measured by the pressure decrease of the system, combines in the peroxidic form initially, then the resulting compound must be considerably less stable than that formed with chlorophyll. Any decomposition of such a compound would appear to be complete before the time of the first estimation in experiment 1, since the peroxide value remains constant thereafter. The irreproducibility evident here between different experiments prevents any conclusion being drawn as to the significance of the absolute values of peroxide obtained, without further detailed examination of the oxidation rate curves of phytol itself.

Photo-oxidations at High Oxygen Pressures In Presence of Adsorbents

Previous work has shown that the unit molecular ratio of gas pressure decrease to pigment concentration is not affected by the presence of carbon dioxide and water. In attempting to confirm that the oxidations previously examined concerned only the formation of a peroxide, experiments were performed with soda lime and/or phosphoric oxide, in a suitable small side tube (see p. 14), on the assumption that carbon dioxide and water would be the most probable products of a more complex oxidation.

In investigating the effect of these adsorbents on the photo-oxidation of chlorophyll, other conditions were maintained the same as in the previous experiments; thus films were illuminated by 'white' light, at 100 mm. oxygen pressure, and at a constant temperature of 25°C. The copper chlorophyll sample first examined here was prepared at a later date than that used in previous experiments, and subsequent investigation of its normal oxidation showed that the unit molecular ratio (p. 68) no longer held. For purposes of comparison, therefore, this normal oxidation is included here, along with the oxidation in the presence of adsorbents.

The sensitivity of the gauge used in all the following experiments was 0·053 mm. per scale division.

Copper Chlorophyll (on thallous bromide)

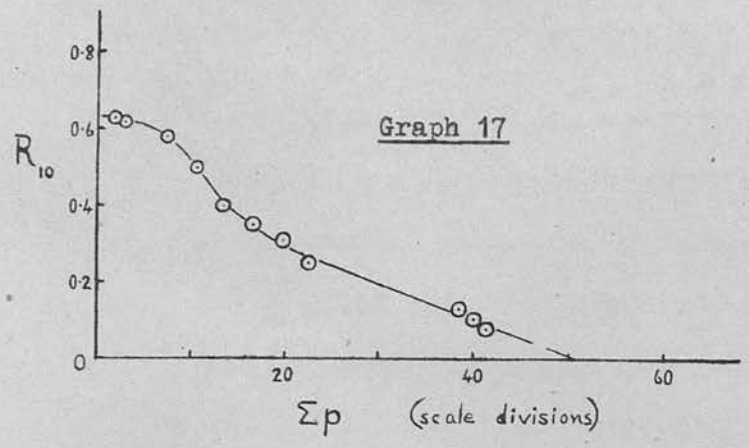
Experimental. 1·54 mg. chlorophyll (copper-containing sample)
0·15 g. thallous bromide
Small vessel
Volume of reaction space = 23·5 ml.

Σp	R_{10}	Σp	R_{10}	Σp	R_{10}	Σp	R_{10}
1·8	0·63	10·5	0·50	19·5	0·31	39·8	0·105
2·7	0·62	13·2	0·40	22·3	0·25	41·2	0·080
7·3	0·58	16·5	0·35	38·5	0·13		

Extrapolated Σp = 50·4 divisions. Molecular ratio = 1·98.

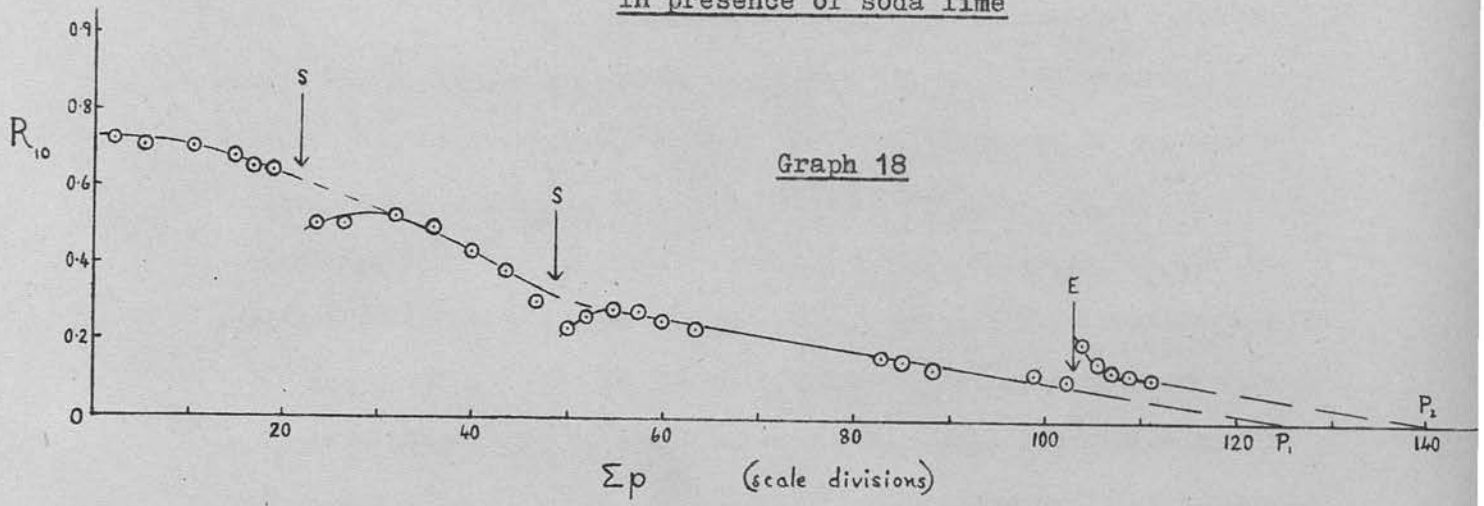
Copper Chlorophyll (on thallous bromide)

Graph 17



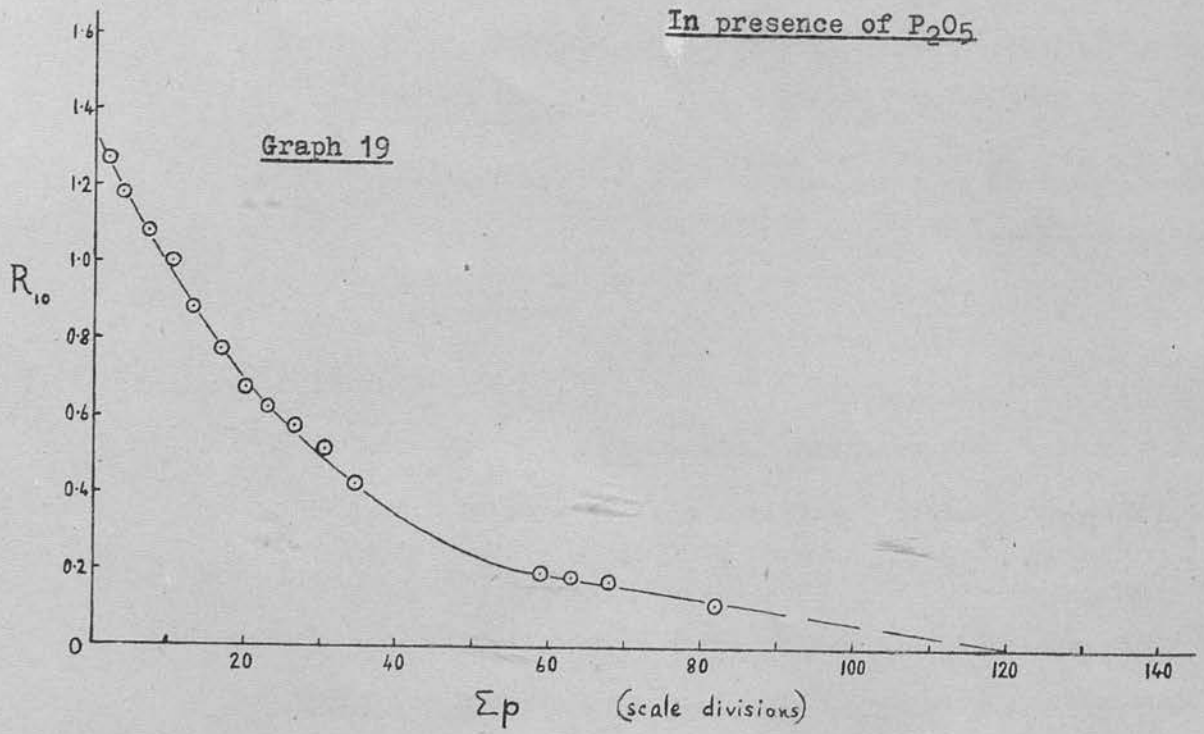
In presence of soda lime

Graph 18



In presence of P_2O_5

Graph 19



The rates obtained are plotted on Graph 17, which then represents the results of a continuous illumination of this sample of chlorophyll.

The Photo-oxidation in presence of Soda Lime

Experimental. 1·54 mg. chlorophyll (copper-containing sample)
0·15 g. thallous bromide
Small vessel with soda lime in side tube
Volume of reaction space = 24·8 ml.

Σp	R_{10}	Σp	R_{10}	Σp	R_{10}	Σp	R_{10}
2·2	0·720	32·0	0·52	57·5	0·275	104·0	0·20
5·4	0·705	35·8	0·49	60·0	0·250	105·5	0·15
10·5	0·700	40·0	0·43	63·5	0·230	107·0	0·13
14·8	0·675	43·6	0·38	83·0	0·155	109·0	0·12
16·7	0·650	46·7	0·30	85·5	0·150	111·0	0·11
18·7	0·640	50·0	0·23	88·5	0·130		
23·6	0·500	52·0	0·26	99·0	0·120		
26·6	0·500	55·0	0·28	102·5	0·105		

Extrapolated Σp (OP_1) = 124·5 divisions. Molecular ratio = 5·15.

At the positions marked S on Graph 18, illumination was interrupted by dark periods in oxygen during which the pressure was unaltered. Acceleration periods are evident after such intervals, but otherwise rates lie on what may be taken as a continuous curve, the form of which is essentially similar to that of Graph 17. Extrapolation of the final linear portion of this curve shows the total pressure decrease to be considerably greater than that found with no adsorbent present. Some gaseous product appears, therefore, to be involved in the reaction. The error in determining the actual value of the total decrease, and hence in finding the molecular ratio of gas pressure decrease to oxidisable material, is greater here as a result of the prolonged illumination (5 days) required, and the difficulty experienced in distinguishing between

rates in the latter stages of the oxidation.

Evacuation of the system (at E on Graph 18) resulted in an increase in rate on re-illumination at 100 mm. oxygen pressure. The linear portion of the rate curve obtained is, as nearly as can be judged, parallel to the extrapolation of the original curve, an effect similar to that observed in normal oxidations.

Soda lime will adsorb in addition to carbon dioxide a considerable quantity of water vapour; if the evacuation be taken as corresponding to a more efficient adsorber, the final extrapolated figure from Graph 18 (OP_2 = 140 div., ratio = 5·79) may be taken as a more accurate value.

The photo-oxidation in presence of phosphoric oxide

Experimental. 1·54 mg. chlorophyll (copper-containing sample)
0·15 g. thallous bromide
Small vessel with phosphoric oxide in side tube
Volume of reaction space = 24·8 ml.

Σp	R_{10}	Σp	R_{10}	Σp	R_{10}	Σp	R_{10}
1·8	1·27	13·1	0·88	26·5	0·57	63·0	0·18
3·8	1·18	16·7	0·77	30·6	0·51	68·0	0·17
7·1	1·08	20·0	0·67	34·7	0·42	82·0	0·11
10·3	1·00	22·9	0·62	59·0	0·19		

Extrapolated Σp = 120 divisions. Molecular ratio = 4·96.

The total pressure decrease in this experiment (Graph 19) is again very much greater than that observed in the absence of an adsorbent. Illumination is continuous, as in the normal oxidation, but the form of the rate curve obtained differs from that of both previous experiments in that no constant rate period is evident;

instead, the rate found on first illuminating is almost double the initial rates of these experiments and decreases relatively rapidly. Such differences may be attributed to the different adsorptive effect of the soda lime [58] and phosphoric oxide [59].

If the extrapolated value 5·79 of the experiment with soda lime were reliable, then approximately one molecule of gas adsorbed by it and not by the phosphoric oxide (5·79 - 4·96) would be produced. This corresponds to one sixth, or 16·7%, of the total pressure decrease — oxygen + water + lime adsorbed constituent.

An experiment in which a film of thallous bromide alone was illuminated in oxygen at 100 mm. pressure in the presence of soda lime, showed no unusual effect which might explain the greatly increased pressure changes observed in these chlorophyll experiments. Thus on first illuminating, a small decrease in pressure of approximately 1 scale division (0·053 mm.) occurred, and thereafter the pressure remained steady (cf. p. 48). A test for bromide ion in the soda lime at the end of these illuminations also proved negative.

The photo-oxidation on glass

Experimental. 1·54 mg. chlorophyll (copper-containing sample)

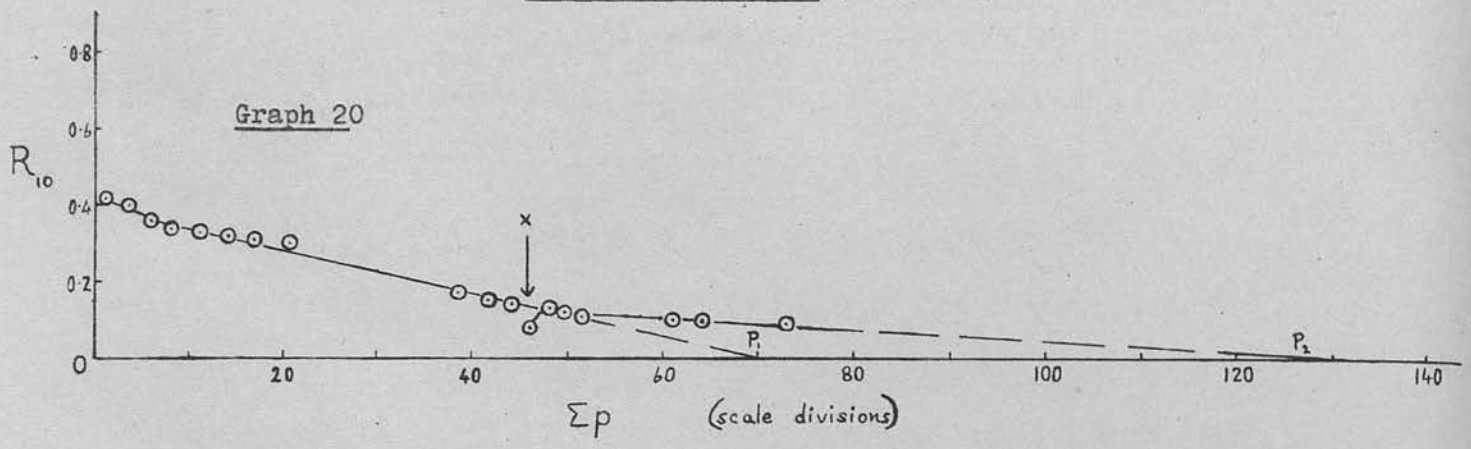
0·15 g. powdered glass (Jena)

Small vessel with side tube (1) empty,
(2) soda lime added

Volume of reaction space (1) 25·0 ml., (2) 24·8 ml.

Copper Chlorophyll (on glass)

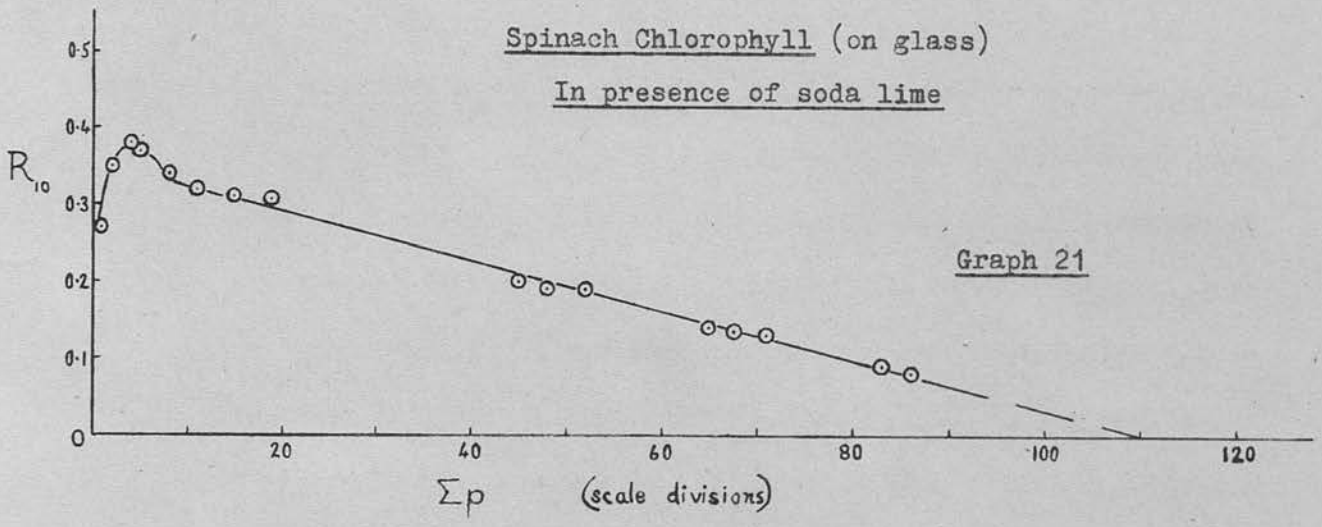
Graph 20



Spinach Chlorophyll (on glass)

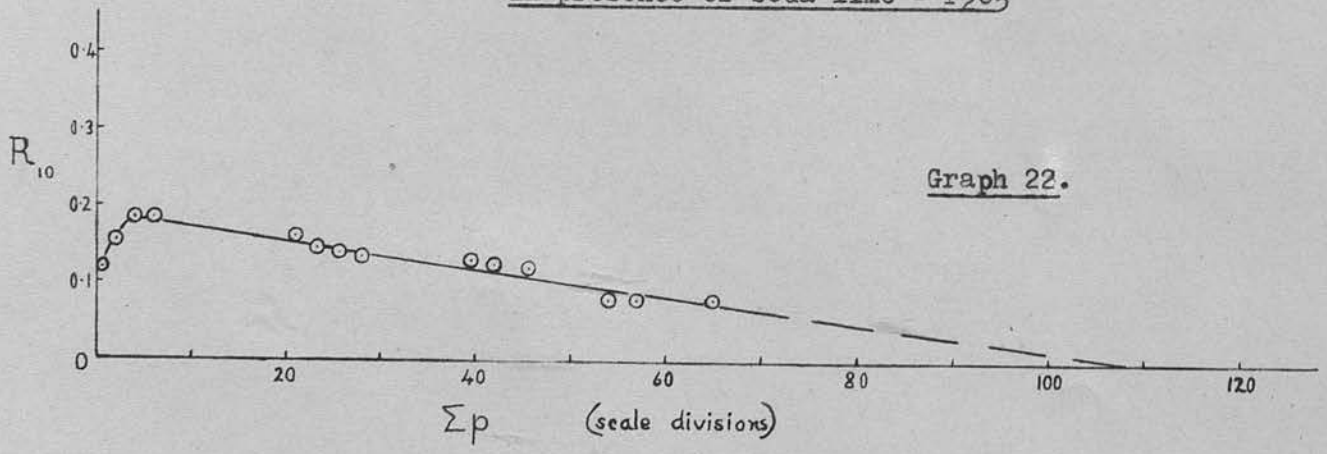
In presence of soda lime

Graph 21



In presence of soda lime - P_2O_5

Graph 22.



Σp	R_{10}	Σp	R_{10}	Σp	R_{10}	Σp	R_{10}
1·2	0·42	14·0	0·32	44·0	0·14	61·0	0·10
3·7	0·40	16·9	0·31	46·1	0·08	64·0	0·10
6·0	0·36	20·6	0·30	48·0	0·13	73·0	0·09
8·0	0·34	38·5	0·17	49·7	0·12		
11·0	0·33	41·5	0·15	51·4	0·11		

1st extrapolated Σp (OP_1) = 70 divisions. Molecular ratio = 2·90.
2nd extrapolated Σp (OP_2) = 126 divisions. Molecular ratio = 5·21.

The molecular ratio obtained by the continuous illumination of this sample of chlorophyll on glass in the absence of any adsorbent (Graph 20), is greater than that of the corresponding experiment on thallous bromide (Graph 17), a result in disagreement with the previous observation (p. 70) that such ratios are equal. Since in either case the ratio observed is greater than unity, it seems possible that this discrepancy is due to the preparation having undergone partial degradation, similar to that observed with the non-stabilised copper-free sample (cf. pp. 41,73). Thus it is a general possibility that the glass, on account of its alkaline nature, is acting in a manner similar to, but much less effective than, the soda lime, and in support of this it may be noted that the final ratio obtained on glass after evacuation and the addition of the soda lime (at X) is in agreement with the first ratio found with the same adsorbent using thallous bromide as substrate. This possibility is further investigated in connection with the photo-oxidation of carotene (p. 104).

The absence of any spectroscopic evidence of degradation with this chlorophyll preparation may be attributed to a masking effect of the copper present as a stabiliser (cf. p. 20). In each experiment the absorption spectrum of the product showed a

markedly depressed red maximum, as observed after all normal oxidations, no other change being evident from that of the unoxidised solution.

Spinach Chlorophyll (on glass)

Similar experiments to the above were carried out on chlorophyll prepared from spinach leaves. Pure chlorophyll obtained in this way has been found to give a pressure decrease equivalent to a molecular ratio of unity, on illumination in oxygen using thallous bromide as substrate.[13c]

The photo-oxidation in presence of soda lime

Experimental. 1·64 mg. spinach chlorophyll
0·15 g. powdered glass (Jena)
Small vessel with soda lime in side tube
Volume of reaction space = 24·8 ml.

Σp	R_{10}	Σp	R_{10}	Σp	R_{10}	Σp	R_{10}
0·7	0·27	8·0	0·34	45·0	0·20	67·5	0·135
2·0	0·35	11·0	0·32	48·0	0·19	71·0	0·130
4·0	0·38	14·9	0·31	52·0	0·19	83·0	0·090
5·0	0·37	18·7	0·31	65·0	0·14	86·0	0·080

Extrapolated Σp = 110 divisions. Molecular ratio = 4·27.

The curve obtained by plotting these rates is shown on Graph 21, which then represents a continuous illumination of this sample of chlorophyll in the presence of soda lime. Removal of the adsorbent after this experiment, followed by evacuation and addition of carbon dioxide, showed no adsorption of this gas by the oxidised film in the dark or on illumination.

The photo-oxidation in presence of soda lime - phosphoric oxide mixture

Experimental. 1·17 mg. spinach chlorophyll

0·15 g. powdered glass (Jena)

Small vessel with soda lime - phosphoric oxide mixture in side tube

Volume of reaction space = 24·8 ml.

Σp	R_{10}	Σp	R_{10}	Σp	R_{10}	Σp	R_{10}
0·3	0·120	21·0	0·160	39·5	0·130	57·0	0·080
2·0	0·155	23·3	0·145	42·0	0·125	65·0	0·080
4·0	0·185	25·6	0·140	45·5	0·120		
6·2	0·185	28·0	0·135	54·0	0·080		

Extrapolated Σp = 108 divisions. Molecular ratio = 5·89.

The form of the rate curve obtained here (Graph 22) is essentially similar to that found in the presence of soda lime only. In both instances a long illumination (4 or 5 days) was required to find the slope of the final linearly descending rate section, and the values obtained for the molecular ratio of gas pressure decrease to chlorophyll must be considered correspondingly less accurate. This is especially so in the present experiment, where rates were low and no large change in them evident over long periods.

A similar examination of this chlorophyll deposited on powdered glass in the absence of any adsorbent, showed a rate curve of the same form as that of Graphs 21 and 22, and a molecular ratio close to three (2·7, 2·9). In general, therefore, it may be concluded that for spinach chlorophyll, as for the copper-containing sample, the molecular pressure decrease to pigment ratio is increased in the presence of such adsorbents.

The high result obtained here in the 'normal' oxidation on glass may have been due to the conversion of the chlorophyll to pheophytin,[27] brought about by conditions in the solid, and facilitated by the long period of illumination involved. Such a change might be expected to occur more readily with this sample of chlorophyll than with the copper stabilised preparation (cf. p. 54). Examination of each film after these experiments did indeed show signs of pheophytin formation, the green colour of the chlorophyll having partly changed to brown, instead of having the normal bleached appearance. No corresponding change was observed with a film of spinach chlorophyll allowed to stand for a similar length of time in oxygen in the dark, but after exposure to 'white' light in vacuo for an equal period a slight 'browning' was evident. This appears to agree with Rabinowitch's observation[60] that pheophytinisation is accelerated by light.

Illumination of the same sample of chlorophyll when deposited on thallous bromide instead of on glass, for a similar period, resulted in an equally marked change in the appearance of the film. The colour of an acetone solution of the product was a definite olive green compared to the pure green of the unoxidised solution, and its absorption spectrum showed not only a marked depression of the red maximum, but also a broadening to longer wavelengths, both observations suggesting the formation of pheophytin. The rates obtained in the early stages of this illumination appeared to be running out to a limit equivalent to a pressure decrease of approximately 1·79 mols per mol of chlorophyll, but on continuing illumination a change in slope was evident. The final extrapolated value obtained after a period

similar to that required in the corresponding glass experiment, gave a molar ratio of 3·93. This departure from the unit molecular ratio associated with pure chlorophyll[13c] appears, therefore, to be due to a degradation of the pigment to pheophytin, begun to a small extent on standing in solution, and accelerated then by conditions in the solid and by illumination, such degradation being observed to different extents on thallous bromide and on glass.

Further examination of products of chlorophyll photo-oxidation:

(1) The gases present after the illumination in oxygen of (a) copper-containing chlorophyll, and (b) spinach chlorophyll, both on thallous bromide in the absence of adsorbents, were tested for carbon dioxide by freezing out in a trap containing a solution of baryta. The formation of a definite precipitate was observed in (a) and accordingly (b) was carried out quantitatively as described on page 26. Using 2 ml. portions of an approximately 0·01 normal solution of baryta, and titrating with a 0·00986 normal solution of hydrochloric acid, the results obtained were as follows:

Difference in titrations = 0·26 ml. 0·00986N hydrochloric acid

$\equiv 1{\cdot}27 \times 10^{-6}$ moles carbon dioxide

Actual pressure decrease observed during illumination

$\equiv 3{\cdot}42 \times 10^{-6}$ moles

$\therefore$ Amount of carbon dioxide as percentage of pressure decrease

= 37·1.

The result of this single estimation shows the amount of carbon dioxide formed during the illumination of chlorophyll to be significant in comparison to the pressure decrease observed.

(2) An approximate estimation of the amount of hydroperoxide formed during illuminations in the presence of soda lime, was made in the case of (a) copper-containing chlorophyll, and (b) spinach chlorophyll.

(a) Immediately after illumination the film of copper chlorophyll on glass, which had been oxidised partly alone and partly in the presence of soda lime (p. 93), was extracted with pure methyl alcohol and measured volumes of the extract estimated for peroxidic oxygen as described on page 28. The result quoted here is the average value of separate determinations, obtained by comparison with the hydrogen peroxide calibration curve, a solution of chlorophyll equivalent in concentration to that remaining on the film being used for comparison in the estimation.

Observed pressure decrease ≡ 169 μg. molecular oxygen

Peroxidic oxygen content of film = 25 μg.

Percentage pressure decrease detected as peroxidic oxygen = 14·8. Comparison of the actual pressure decrease with the total extrapolated value (Graph 20), shows the chlorophyll to have been approximately 57% oxidised.

(b) A similar extraction and estimation of the film of spinach chlorophyll on glass after oxidation in the presence of soda lime, gave the following results -

Observed pressure decrease ≡ 197 μg. molecular oxygen

Periodic oxygen content of film = 30 μg.

Percentage pressure decrease detected as peroxidic oxygen = 15·2. Oxidation in this case is seen from the rate curve (Graph 21) to be approximately 80%.

The low percentages of peroxidic oxygen detected here are in marked contrast to the values obtained previously after normal oxidations (pp. 81-87). With both these samples of chlorophyll, however, the molecular ratio of pressure decrease to oxidisable material in the absence of adsorbents is greater than unity. Illumination of the copper chlorophyll in the absence of any adsorbent, and estimation when the observed pressure decrease was equal to approximately the same percentage of unit molecular decrease (i.e. 51%) as in the previous determinations, gave a peroxide percentage of approximately 70. From this it may be concluded that in the initial stages at least, the reaction taking place is still the absorption of oxygen to form mainly a hydroperoxide. The small amounts of peroxidic oxygen detected in the present experiments may, therefore, indicate either that the hydroperoxide formed initially has decomposed as a result of the long illumination required, or that by the removal of some gaseous product the reaction has taken a different course. These points will be referred to further in the subsequent general discussion.

Carotene

Previous work on the photo-oxidation of the carotenoid fraction, when adsorbed on films of thallous bromide, has shown a molecular ratio of pressure decrease to oxidisable material of unity, and a 'reversal' effect similar to but smaller than that observed with chlorophyll. In view of this similarity in the photo-oxidation characteristics of the two pigments when adsorbed on thallous bromide, it was of interest to see whether the

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resemblance held when an inert substance such as powdered glass was used as film substrate, and also whether the addition of adsorbents had any effect on the pressure changes observed.

In the following experiments the carotenoid fraction obtained from the separation of the copper-containing commercial chlorophyll paste (p. 18) was used, the reaction being carried out at 100 mm. oxygen pressure, using 'white' light illumination, at 25°C. In calculating the molecular ratio of pressure decrease to oxidisable material, the molecular weight of the carotenoid fraction was taken as that of carotene itself, 536. The two most common compounds of the 'yellow fractions' obtained from leaf extracts are xanthophyll $C_{40}H_{54}(OH)_2$ and β carotene $C_{40}H_{56}$, the former being present in greater concentrations than the latter in the higher plants. The present solution was shown, however, to contain very little xanthophyll, a test given by Spoehr[61] to distinguish xanthophyll by the appearance of a green colour on dissolving in warm ethyl alcohol containing concentrated hydrochloric acid, proving negative. For the present purposes, therefore, very little error will be introduced by taking the composite molecular weight of the fraction as that of β carotene itself.[62]

The photo-oxidation on glass

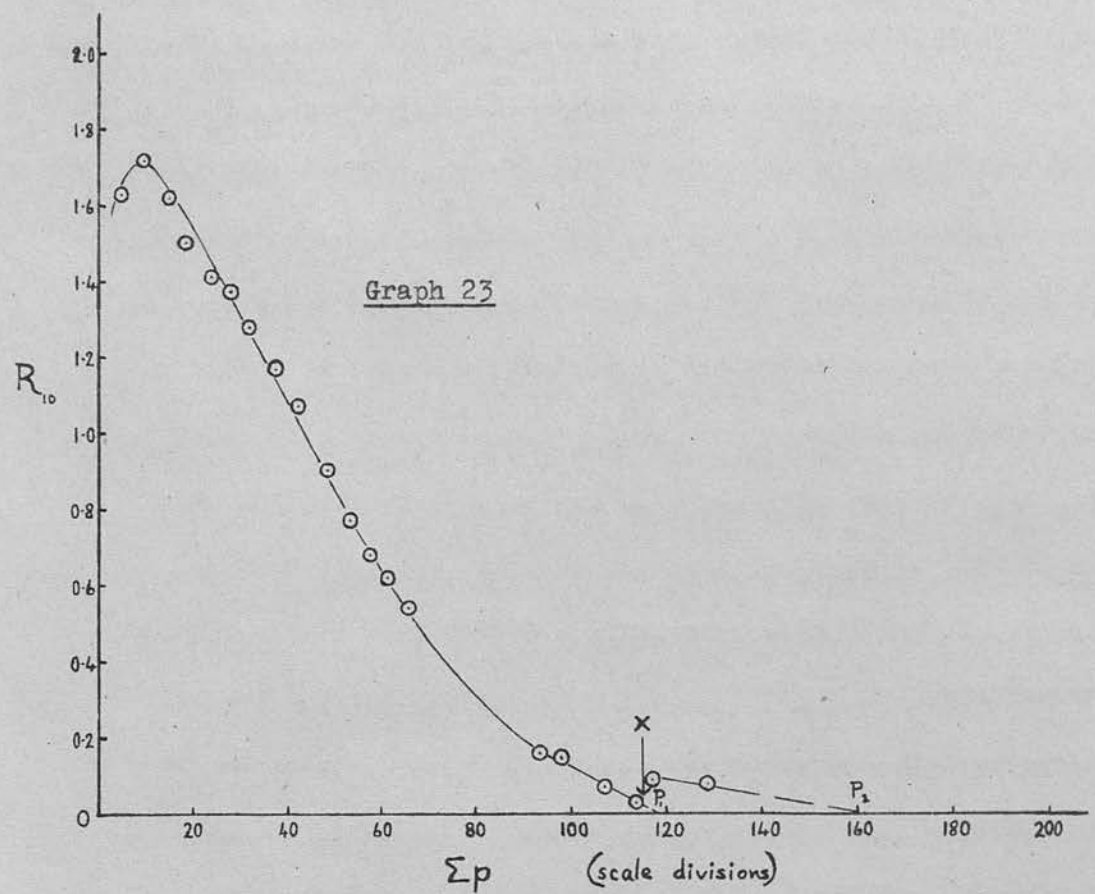
Experimental. 1·57 mg. carotene

0·15 g. powdered glass (Jena)

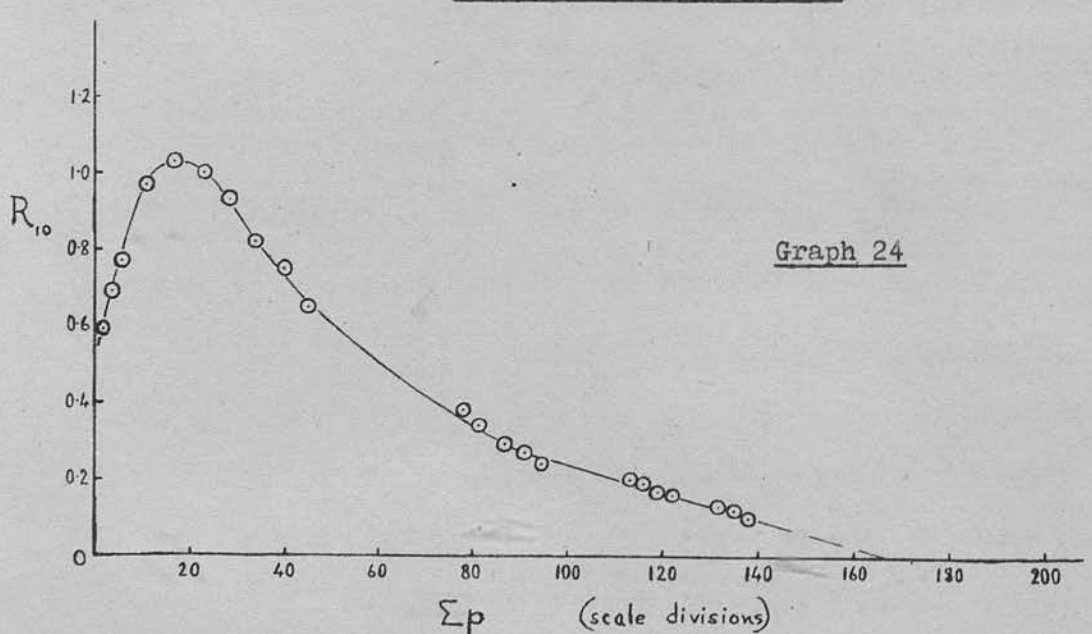
Small vessel with side tube (1) empty
(2) soda lime added

Volume of reaction space (1) 25·0 ml. (2) 24·8 ml.

Carotene (on glass)



In presence of soda lime



Σp	R_{10}	Σp	R_{10}	Σp	R_{10}	Σp	R_{10}
4·7	1·63	28·1	1·37	53·6	0·77	98·0	0·15
9·8	1·72	32·1	1·28	57·8	0·68	107·0	0·07
15·0	1·62	37·6	1·17	61·7	0·62	113·8	0·03
18·5	1·50	42·7	1·07	65·9	0·54	117·2	0·09
24·0	1·41	48·6	0·90	93·3	0·16	128·8	0·08

1st extrapolated Σp (OP_1) = 118 divisions.
Molecular ratio = 2·88.

2nd extrapolated Σp (OP_2) = 160 divisions.
Molecular ratio = 3·87.

The rates obtained in this experiment are shown in Graph 23, which then represents a continuous 'normal' oxidation of carotene on glass.

Addition of soda lime at the position X on the graph, followed by evacuation, resulted in an increase in the rate of pressure decrease on re-illumination at the same oxygen pressure. The slope of the curve obtained then differs from that of the final linear section of the original curve, an effect similar to that observed with chlorophyll after the same treatment (Graph 20).

<u>The photo-oxidation in presence of soda lime</u>

Experimental. 1·57 mg. carotene
0·15 g. powdered glass (Jena)

Small vessel with soda lime in side tube

Volume of reaction space = 24·8 ml.

Σp	R_{10}	Σp	R_{10}	Σp	R_{10}	Σp	R_{10}
1·5	0·59	28·6	0·93	86·6	0·29	121·8	0·16
3·5	0·69	33·9	0·82	90·7	0·27	132·0	0·13
5·5	0·77	40·0	0·75	94·0	0·24	135·0	0·12
10·7	0·97	45·0	0·65	113·0	0·20	138·0	0·10
16·8	1·03	77·9	0·38	116·0	0·19		
23·0	1·00	81·1	0·34	119·0	0·17		

Extrapolated Σp = 166 divisions.

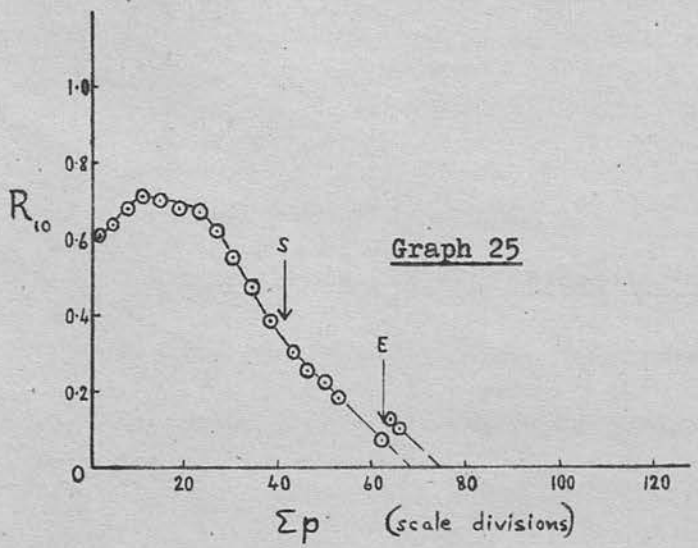
Molecular ratio = 4·02.

Comparison of the results of these two experiments shows that for carotene on glass, as for chlorophyll, an increase in the molecular ratio is obtained in the presence of an adsorbent; it appears, therefore, that here also the pressure decreases observed normally refer not simply to an uptake of oxygen by the pigment, but to an uptake accompanied by the liberation of some resultant gas. The form of the rate curve obtained is essentially the same in both experiments (Graphs 23 and 24), consisting of an induction or acceleration period, and a decreasing rate section apparently tending first to a limit equivalent to a molecular ratio of

approximately two, but gradually changing slope, becoming almost linear again at comparatively low rates, and giving a final molar ratio of about three alone and of four in the presence of soda lime.

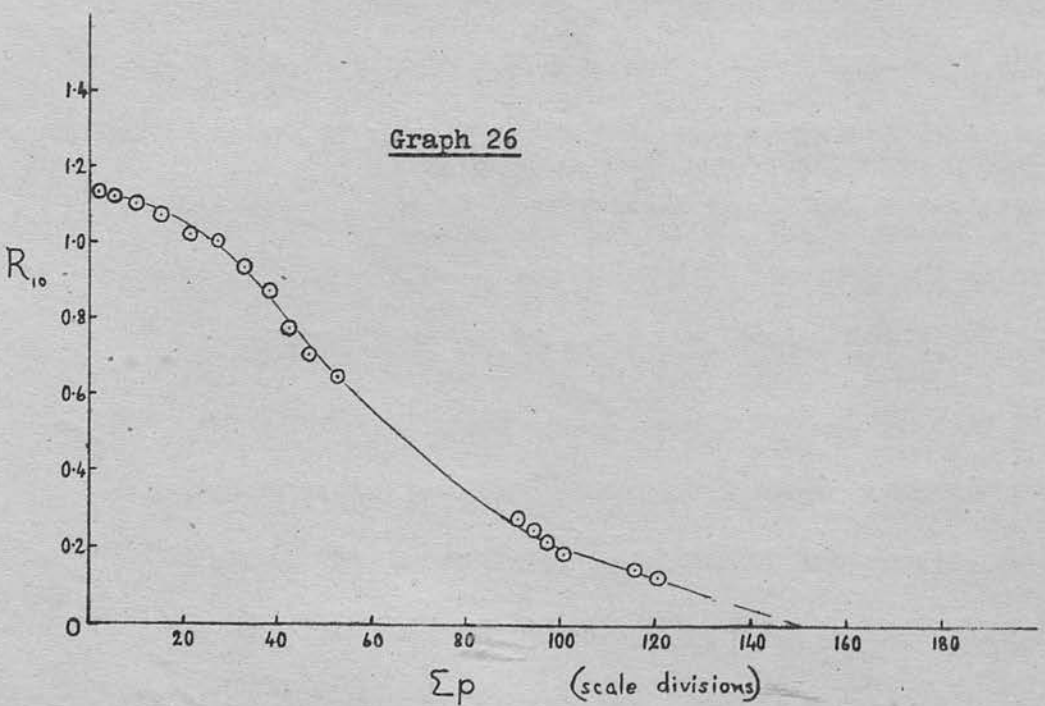
If, as such behaviour suggests, the carotenoid molecule is breaking up as a result of the prolonged illumination required in these experiments, then a possible oxidation product which is volatile is carbon dioxide. Direct examination of the effect of this gas on a film of powdered glass (Jena) with no pigment present showed that adsorption occurred in the dark, but to such a small extent (0·05 mm. at 3 mm. CO_2 pressure) as to be negligible in comparison to the amounts which must be involved in view of the difference between the ratios with and without soda lime present, or which might explain the high normal ratio observed on glass compared to that reported for similar conditions using thallous bromide. No further adsorption of carbon dioxide occurred in the presence of water vapour. With a film of carotene on powdered glass, a dark adsorption of the same magnitude was observed with dry carbon dioxide at a similar pressure. At 25 mm. pressure a further small adsorption (0·08 mm.) occurred before steady conditions were reached, no change resulting then on illumination. Since presumably at this pressure the film was saturated with carbon dioxide, any produced during the oxidation of the pigment must remain unadsorbed and an alteration in the

Carotene (on thallous bromide)



Graph 25

In presence of soda lime



Graph 26

molecular ratio might be expected; in practice, however, no such change was observed.

The question was further investigated by carrying out parallel experiments with this sample of carotene on thallous bromide instead of on glass.

The photo-oxidation on thallous bromide

Experimental. 1·10 mg. carotene
0·15 g. thallous bromide
Small vessel
Volume of reaction space = 23·5 ml.

Σp	R_{10}	Σp	R_{10}	Σp	R_{10}	Σp	R_{10}
1·5	0·61	19·0	0·68	38·2	0·38	62·4	0·07
4·6	0·64	23·2	0·67	43·0	0·30	64·0	0·12
7·6	0·68	27·0	0·62	46·2	0·25	66·0	0·10
10·7	0·71	30·3	0·55	50·0	0·22		
15·0	0·70	34·4	0·47	53·0	0·18		

Extrapolated Σp = 68 divisions.
Molecular ratio = 2·22.

At the position S on graph 25, illumination was interrupted by a dark period in oxygen during which the pressure was unchanged. No marked alteration in rate is evident after this interval, and the complete curve obtained is similar in form to that observed using glass as substrate. The molecular ratio obtained by the extrapo-

lation of the final linear section of this curve is smaller than the corresponding value on glass (2·88), but is still greater than unity, a result which may possibly be due to a partial degradation of the pigment similar to that observed in the case of chlorophyll. If this is so, then it appears that such degradation can take place even in solution in the dark.

The normal 'reversal' effect is observed in this experiment after evacuation (at E on Graph 25), the slope of the curve obtained then on re-illumination at the same oxygen pressure being equal to that of the original curve.

The photo-oxidation in presence of soda lime

Experimental. 1·10 mg. carotene
0·15 g. thallous bromide
Small vessel with soda lime in side tube
Volume of reaction space = 24·8 ml.

Σp	R_{10}	Σp	R_{10}	Σp	R_{10}	Σp	R_{10}
1·8	1·13	27·1	1·00	52·7	0·64	116·0	0·14
5·0	1·12	33·0	0·93	91·2	0·27	120·8	0·12
10·0	1·10	38·5	0·87	94·8	0·24		
15·0	1·07	42·3	0·77	97·6	0·21		
21·2	1·02	46·6	0·70	101·0	0·18		

Extrapolated Σp = 150 divisions. Molecular ratio = 5·17.

The same general conclusion may be drawn from a comparison of the results of these two experiments on thallous bromide (Graphs 25 and 26) as from the corresponding ones using powdered glass as substrate, namely that some gaseous product is involved in the photo-reaction, the removal of which by an adsorbent gives an

increased molecular ratio. In this case the increase observed is equivalent to three molecules, while in the glass experiments a difference of only one was observed. While this again seems to point to some adsorptive effect of the glass substrate, the fact that in the presence of a strong adsorbent the final ratios obtained on the two supports are different, indicates that the reaction is complex and occurs to a different extent under slightly different conditions.

Examination of products of carotene photo-oxidation:

(1) After illumination in oxygen each film was seen to be considerably bleached, and on extraction with organic solvents gave a very pale yellow, almost colourless, solution.

(2) Tests for peroxide and aldehyde carried out on the gases present after these experiments, and on direct aqueous extracts of the films, were all negative, the solutions obtained being neutral.

(3) The gases present after the oxidation on thallous bromide in the absence of adsorbents (at point E on Graph 25), were frozen out in a trap containing 2 ml. of an approximately 0·01 normal solution of baryta, and the slight precipitate formed was estimated as described on page 26, using a 0·00986 normal solution of hydrochloric acid. The results obtained were as follows -

Difference in titrations = 0·08 ml. 0·00986N hydrochloric acid

$\equiv 0{\cdot}39 \times 10^{-6}$ moles carbon dioxide

Actual pressure decrease observed during illumination

$\equiv 4{\cdot}24 \times 10^{-6}$ moles

∴ Amount of carbon dioxide as percentage of pressure decrease

$\equiv 9{\cdot}2$.

The difference in the titrations here is small and not outside the bounds of experimental error, as was shown by control experiments. It may therefore be concluded that very little, if any, carbon dioxide is formed during the illumination of carotene in oxygen.

(4) The amount of hydroperoxidic oxygen present after the illumination of carotene in oxygen, using glass films, was estimated by means of ferrous ammonium thiocyanate reagent, using the method described for chlorophyll on page 28.

Direct extraction with reagent of the film, oxidised mainly in the absence of an adsorbent (p. 101), and estimation by comparison with the hydrogen peroxide calibration curve, showed that only 5% of the observed pressure decrease was detectable as peroxidic oxygen. By comparison of the actual pressure decrease with the final extrapolated value (Graph 23) the film could be taken as 81% oxidised.

A similar estimation after the oxidation on glass in the presence of soda lime, gave the hydroperoxide content as 10% of the observed pressure decrease, where the extent of oxidation was estimated by extrapolation (Graph 24) to be 83% of the total.

A film of carotene oxidised in the absence of adsorbents to approximately 60% of the total pressure decrease, showed a greater hydroperoxide content than in the other two experiments, the actual value obtained being equivalent to 25% of the observed pressure decrease.

The percentages of peroxidic oxygen quoted here have no more than a relative significance, and can be taken merely as indicators of the state of decomposition of the oxidised carotene: thus it is

seen from these results that the greater the extent of oxidation, i.e. the longer the illumination, the less hydroperoxidic material remains. A more extensive investigation of the amounts of hydroperoxide present at earlier stages of the oxidation, similar to that carried out for the chlorophyll molecule, and with particular reference to the addition of the first molecule of oxygen, would be necessary before any more definite conclusions could be drawn from these results.

In considering the possibility that the rate 'reversal' on evacuation observed in the normal photo-oxidation of chlorophyll and carotene, is due to the removal of some retarding product or reaction, in which an increase in pressure must be concerned (p. 6), the scheme of reactions taking place may be represented in relation to oxygen uptake by

$$M + nO_2 \longrightarrow M'(O_2)_n + A \qquad (1)$$

$$A + O_2 \longrightarrow B + nP \qquad (2)$$

where $n > 1$, M represents the chlorophyll or carotenoid molecule, A and B are solids of inappreciable vapour pressure, and P is a volatile non-adsorbed gaseous product. The mechanism of (1) would of course involve separate or consecutive reactions, but the overall pressure decrease observed would be $(n+1)-n = 1$ volume, i.e. preserving the unit molecular ratio.

To explain the 'reversal' of this oxidation, the product A must have a vapour pressure which, though inappreciable, is such that evacuation to a pressure of less than 0·001 mm. may remove it. On continuing the illumination the retarding reaction (2) is now initially absent and the rate will show an increased value. Such

'reversal' will be the less the longer the illumination and the less the amount of A. Further, the proportion of A to M in such consecutive reactions will reach an equilibrium value, dependent on the relative velocity coefficients and the light absorption; conditions will then be the same as before evacuation and the slope of the rate curve equal to that of the original.

If this explanation of the essential features of the photo-oxidation both of chlorophyll and of carotene is correct, then it should theoretically be possible to isolate some substance A, the oxidation of which would result in an increase in pressure. Attempts to do this were made as follows -

(a) Chlorophyll. A film of copper chlorophyll on thallous bromide was illuminated at 100 mm. oxygen pressure until a pressure decrease of approximately 20% of the total was obtained, experiment having shown that maximum 'reversal' occurs in this stage of the reaction.[46] After oxidation the system was pumped out to a pressure of less than 0·001 mm. through a liquid oxygen trap containing thallous bromide crystals; these were transferred to a reaction vessel and made to adhere to one face of it by the addition of a drop of ether. The air in the system was then displaced as far as possible by pure oxygen, without reducing the pressure below 100 mm., and the thallous bromide illuminated when steady conditions had been reached in the dark. No change in pressure was observed, although re-illumination of the partially oxidised chlorophyll film showed the normal 'reversal' effect. In view of the small pressure decrease normally observed with thallous bromide alone in oxygen (p. 48), it is possible that the absence of any appreciable pressure change under these

conditions was due to the simultaneous occurrence of this decrease with the increase represented by equation (2), though such an equality of two independent processes seems improbable.

(b) Carotene. A similar procedure was adopted with carotene; thus after suitable oxidation and trapping out of the products, the thallous bromide crystals were transferred to a reaction vessel and a drop of water added in this case to form the film. The water was allowed to distil over in the dark into a phosphoric oxide tube, and after steady conditions had been reached, the film was illuminated in oxygen. Again no increase in pressure was observed which might be taken as positive evidence of the oxidation of the compound A.

GENERAL DISCUSSION AND CONCLUSIONS

The results obtained in the early part of the present examination of chlorophyll and its related compounds, appeared at first to support the theory previously advanced that such pigment oxidations were reversible; so many other factors are now revealed by the study of these systems, however, that this view can no longer be upheld. In consideration of this development, it may perhaps be best to discuss the present findings from the point of view of the reversible oxide theory first, and then to consider them more generally in the light of the subsequent observations. Consideration will be given at the same time to the conditions under which these oxidations were carried out, and to their effect on the experimentally observed features of the reactions.

The photo-oxidation of chlorophyll has been shown to take place with a unit molecular ratio of pressure decrease to pigment concentration, and with an increased rate of pressure change after pressure reduction; on the basis mainly of these observations, the scheme of reactions given on page 4 has been proposed.[13] These results may be taken to be essentially the same for intact chlorophyll preparations, whether adsorbed on the accelerating substance thallous bromide or on an inert substrate such as powdered glass.

With regard to the reversible nature of this autoxidation, it may first be noted that the system represented by equations (5) and (6) of the scheme, namely

$$Chl + O_2 \rightleftharpoons Chl.O_2$$

is directly analogous to that involved in the formation of calcium carbonate -

$$CaO + CO_2 \rightleftharpoons CaCO_3$$

With a univariant system of this nature, consisting of a gas in contact with two distinct solid phases, according to the phase rule equilibrium is reached at a definite gas pressure at a given temperature. The value of this equilibrium or dissociation pressure is independent of the absolute amounts of the reactants present. At carbon dioxide pressures greater than this limit, formation of carbonate takes place at the expense of calcium oxide, while at lower pressures carbonate dissociates until this equilibrium pressure is restored, or until all the carbonate phase has disappeared.

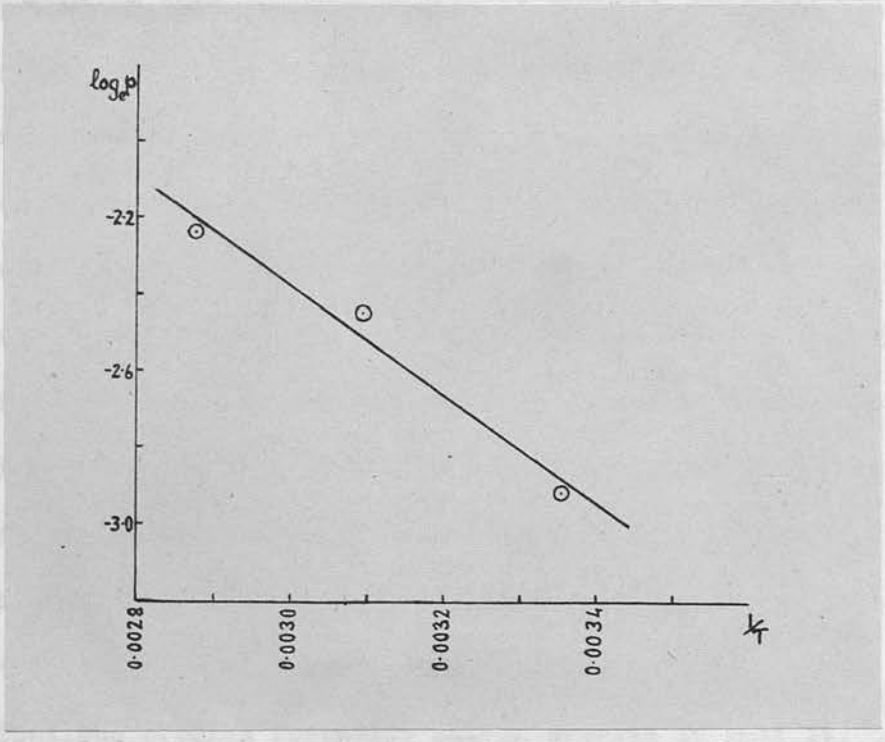
It was first believed that the behaviour of the chlorophyll-oxygen system under the present conditions indicated the establishment of an equilibrium of this nature. Thus it has been shown that for any given temperature there exists a pressure below which in general practically no oxidation takes place. At oxygen pressures higher than this the 'reversible peroxide' phase is apparently stable, no dissociation in the dark being observed and the system appearing unchanged on re-illumination, except in those instances where a retardation in rate was evident. Also, although all attempts to observe the dissociation of any reversible peroxide at pressures below this limiting value were unsuccessful, yet after such pressure reduction the rate of oxidation on recommencing illumination and the total gas pressure decrease both indicated some reversal to have occurred.

The value of this pressure limit is small but significant, and may be taken for pure (spinach) chlorophyll as equal to 0·054 mm. at 25°C (p. 56). It is of interest to consider the energy values for the oxidation which would correspond to an equilibrium pressure of this value, and to compare them with the

corresponding figures for other reversible oxidations. The following table summarises the results obtained in the investigation of the limit (p) at different temperatures (t);

t (°C)	T (°A)	$\frac{1}{T}$	p (mm.)	$\log_e p$	$\Delta F = RT \log_e \frac{p}{760}$ (k. cal.)
25	298	0·003356	0·054	-2·92	-5·7
50	323	0·003096	0·086	-2·45	-5·8
75	348	0·002874	0·107	-2·24	-6·1

where ΔF is the free energy change.



The slope of the linear plot of $\log_e p$ against $\frac{1}{T}$ corresponds to a heat of dissociation of 2·8 k. calories.

In the case of rubrene a dissociation pressure of 5 mm. at 16°C was observed for the oxidation, corresponding to $\Delta F = -2·9$ k. calories, while by direct calorimetric measurements the heat of dissociation was found to be 23 k. calories. For hemoglobin the values reported for the heat evolved in the combination with one

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gram molecule of oxygen vary from 5·35 k. calories at 38°C to 15·0 k. calories at 34°C.[65] These low values are in contrast to the ones associated with irreversible peroxide formation, the heat evolved when one gram molecule of oxygen is introduced into the ethylene bond being about 50 k. calories.

The limiting pressure observed for pure (spinach) chlorophyll is apparently the same when adsorbed on glass as on thallous bromide, although for the other preparations examined, this agreement was not observed. While the general behaviour of the two systems in this pressure region is similar, evidence points to a retarding effect in experiments using thallous bromide which is not present with the inert material. Thus whereas with films of chlorophyll on glass, rates appear to be unaffected by dark intervals in oxygen, using thallous bromide they are markedly decreased (pp. 45-55). No pressure change is involved and the effect is removable by evacuation, only occurring during the early illuminations of a film. At high oxygen pressures also (e.g. 100 mm.) rates are apparently reduced after such intervals (p. 69) though again only where oxidation has not proceeded far (i.e. less than 40%).

Thallous bromide resembles silver bromide in that it decomposes in blue light in the presence of a bromine atom acceptor such as gelatin with the formation of a thallium latent image,[66] and it is a general possibility that chlorophyll and carotene, by virtue of their unsaturated natures, enable this to occur. That such is not the case is indicated by the fact that no effect of added water vapour or carbon dioxide has been observed, either on the rate of pressure decrease or on the final gas

uptake[13]; under conditions where such must otherwise have produced an additional pressure decrease, according to the following mechanisms -

(a) (1) $2Tl + 2H_2O = 2TlOH + H_2$

(2) $2Tl + H_2O + CO_2 = Tl_2CO_3 + H_2$

(b) In presence of oxygen

(3) $4Tl + O_2 + 2H_2O = 4TlOH$

(4) $4TlOH + 2CO_2 = 2Tl_2CO_3 + 2H_2O$

At the same time no evidence of free bromine formation was obtained, either after a typical oxidation on thallous bromide or after illumination of the substrate itself, in vacuo or in oxygen. The small but appreciable pressure decrease observed on illumination in oxygen in the absence of pigment (p. 48), can only then imply the existence in the thallous bromide of a number of thallium atoms, formed perhaps during its preparation, even though precipitation was carried out in the presence of excess bromine ions. A trace of water trapped in the film reacting with this thallium in the presence of oxygen, according to equation (3), would explain the observed pressure decrease; the progressively decreasing apparent reversibility of this effect could then be accounted for by an opening up of the film by evacuation, with consequently increased accessibility to any water and thallium remaining after such reaction. The part played by light in this oxidation is difficult to discern; it may however be connected with the size of the aggregate of thallium atoms, which will be altered by light action, on the general view of latent image formation as advanced by Gurney and Mott[67], or by the later theory of Mitchell[68] concerning the migration of vacant anion sites.

Adsorption of pigment on the thallous bromide crystals must be expected to reduce their direct availability to oxygen. It is possible, therefore, that the pressure decrease observed with the substrate alone becomes then of negligible magnitude in comparison to the reaction with the pigment. This would appear to be borne out by the agreement observed in the pressure limits for pure (spinach) chlorophyll when adsorbed on thallous bromide and on glass. At high oxygen pressures the effect may be neglected without appreciable error in comparison to the total pressure changes involved.

Since the oxidation using thallous bromide is a photo-sensitised one, the retarding influence of dark intervals in oxygen suggests that the surface of the substrate which supplies the energy for the reaction is in some way deactivated. Such a removal of the 'active spots' of energy transfer would appear to be reversible, and to be greatest at high oxygen pressures - an effect which would, partly at least, account for the fact that rates at 100 mm. are not in proportion to those observed at low pressures (< 0·5 mm.). In general an alteration in the rate of supply of energy should not affect the total pressure decrease or the value of the equilibrium pressure for a reaction, though it might result in an apparent tending of rates to limits respectively lower and higher than the true values. Such behaviour has indeed been observed in the present work (pp. 45-55, 69). After a certain time interval or period of illumination, however, steady surface conditions appear to be established, since this retardation is no longer observed and reproducible results are obtained.

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The existence in thallous bromide of a conductivity level, similar to that in silver bromide, in which electrons are mobile,

makes it almost certain that the absorption of blue light by the bromine ion results in the production of an electron in this level. Thus -

$$Br^- + h\nu \longrightarrow Br + \varepsilon_1 \qquad (1)$$

If the product of the oxidation of pigment molecules is regarded as $[A^+ O_2^-]$ [70], then the accelerating effect of thallous bromide on the reaction may be considered as resulting from the following reactions

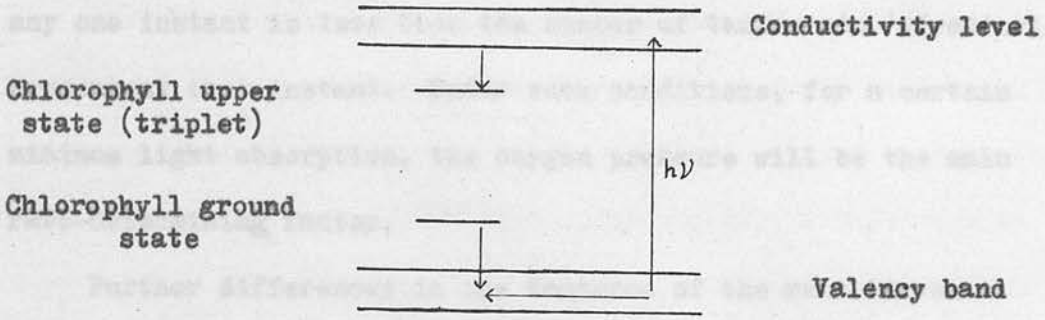
$$A \longrightarrow A^+ + \varepsilon_2 \qquad (2)$$

$$Br + \varepsilon_2 \longrightarrow Br^-$$

$$O_2 + \varepsilon_1 + A^+ \longrightarrow [A^+ O_2^-]$$

where A is the molecule of pigment. In consideration of the photoconducting [71] and photosensitising [72] properties of chlorophyll, it seems probable that reaction (2), the expulsion of an electron from the molecule, requires the absorption of light; A^+ is probably then a tautomeric triplet state of the molecule. Such a long-lived energy-rich state has been postulated for chlorophyll in explanation of the phosphorescence and reversible bleaching which have been observed [73]. Since triplet states arise from the decoupling of the spins of two electrons so that they are unable to share a normal bonding orbital, the accelerating effect of the thallous bromide, expressed in the above reactions, may be interpreted as due to an increase in the lifetime of the metastable state by the expulsion of one of these electrons into the conducting lattice of the supporting solid.

An alternative presentation of the process may be visualised as follows -



where an electron is raised from the valency band of the thallous bromide to its conductivity level by the absorption of light, the positive hole thus produced migrates, its movement being neutralised by an electron from the chlorophyll, and the electron in the conductivity level is 'trapped' by the upper chlorophyll level. The net result of this change is

$$Chl + h\nu \longrightarrow Chl^{*}$$

although the light is absorbed by the thallous bromide.

In the absence of a conducting lattice the absolute light absorption will be smaller and a slower rate of reaction result. This effect is evidenced by the experiments with powdered glass (p. 71) at high oxygen pressures (e.g. 100 mm.), where microscopic examination has shown the grains to be approximately the same size as those of thallous bromide. At low pressures, however ($< 0{\cdot}5$ mm.) no such acceleration was apparent on thallous bromide, rates of reaction with chlorophyll being then substantially the same on either substrate. The effectiveness of such small concentrations of oxygen may be taken as further evidence of the existence of a long-lived activated state of the chlorophyll molecule; the absence of any marked photosensitisation by the thallous bromide would then seem to indicate that, at these pressures, the number of oxygen molecules directly in contact with the chlorophyll at

any one instant is less than the number of tautomeric 'diradicals' present at that instant. Under such conditions, for a certain minimum light absorption, the oxygen pressure will be the main rate-determining factor.

Further differences in the features of the rate curves obtained using thallous bromide and glass, might be expected to arise as a result of effects such as diffusion. The reaction on thallous bromide is a photosensitised one, and for oxidation to take place at the active centres of its surface, oxygen must first diffuse through the thickness of the adsorbed material. Before illumination in all experiments, it was necessary to allow some considerable time for steady temperature conditions to be reached after the addition of oxygen, and the film on illumination may thus be regarded as at a uniform concentration of oxygen. Oxidation taking place at the thallous bromide surface then will not show as a pressure change, until an oxygen concentration gradient is set up, resulting in diffusion of the gas through the film; acceleration or induction periods might, therefore, be expected. With films of equal thickness on glass such effects should be much less obvious, since oxidation will take place mainly at the outer surface and diffusion be relatively less important. This is indeed the case in the present work, induction periods being less evident in experiments with glass than in those with thallous bromide. The rate at which diffusion occurs will be determined by the oxygen pressure, the film thickness (see p. 60), and also possibly by the nature of the oxidation product.

Results at low oxygen pressures are not reproducible in the sense that in general higher rates of pressure decrease are shown

for the same pressures after interruption of illumination by evacuation. This non-reproducibility is apparent in oxidations using powdered glass as substrate as well as in those with thallous bromide, and thus appears to be independent of any diffusion or deactivation effects connected with the photosensitising action of the latter. Such behaviour suggests that oxidation is accompanied by the production of some resultant gas or gases. The removal of any such retarding product would explain the increased rates observed on evacuation, and in the same way the presence of a suitable adsorbent should eliminate this anomalous effect. Experimentally, however, it was observed that the addition of phosphoric oxide to the reaction medium at low pressures did not prevent the apparent retardation (p. 62); on the other hand it may be noted that the rate curves obtained then differed from the normal ones for the same pressures, no marked pressure limit being apparent.

Alternatively it is possible that some material is introduced with the chlorophyll, of small amount and volatile slowly at low pressures. Certain properties of this pigment indicate that it forms a complex with water or other hydrogen-bonding substances[44]; thus it has been shown that both the fluorescence intensity and the absorption spectrum of its solutions in hydrocarbons depend on the presence or absence of traces of such substances. Since water in photosynthesis is a reducing agent, and photochemical bleaching of sensitising dyes is in some cases greatly accelerated by the presence of traces of reducers[74], it may be that the rate of oxidation of chlorophyll is altered in this way by the presence of adsorbed water traces, or on evacuation by the production of water-

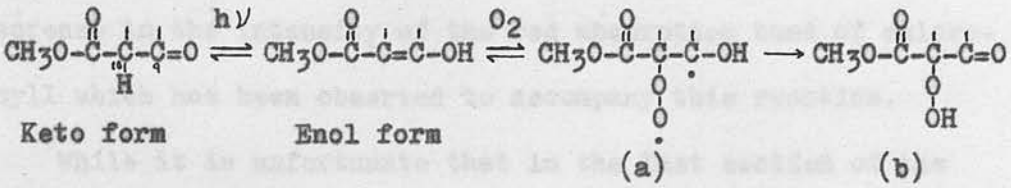
free molecules, which presumably would have different properties from the complex. Further, since a tendency has been observed for the absorption spectrum and intensity of fluorescence to approach those characteristic of unactivated solutions with rise in temperature, indicating the volatilisation of the adsorbed water traces, any rate differences brought about by evacuation might be expected to be accentuated at higher temperatures. Comparison of the rate curves at different temperatures in this pressure region, does show the increase in rate with evacuation to be more marked at the higher temperatures used; in the presence of phosphoric oxide the difference is even more definite, suggesting that the effect is indeed due to a removal of water.

It is of interest to note that the above-discussed effects observed with the chlorophyll-oxygen system at pressures near the critical limit - for example the appearance of apparent equilibria where the initial oxygen pressure is considerably greater than the dissociation pressure, the retardation of the reaction by its products, acceleration after interruption, and the increase in rate on approaching equilibrium - are similar to those reported for the parallel case of the formation of calcium carbonate.[75] Such effects have been interpreted as arising from the formation of crystals of different sizes under different conditions, the dissociation pressure being a function of particle size; thus with calcium oxide and carbon dioxide at high pressures there is supersaturation so that nuclei are readily formed, giving calcium carbonate in small crystals with a dissociation pressure greater than the true value. While the establishment of apparent equilibria of this nature is a possible explanation of certain of the present observations, it is obvious that in this case several other factors are involved and that an interpretation on this basis would not be complete.

The general chemistry of the chlorophyll molecule indicates that although it contains several groupings which are possible centres of oxidation (see p. 6), one of these - the hydrogen atom at carbon atom 10 - is particularly reactive, while removal of the central magnesium atom by dilute acids produces two further readily oxidisable positions. Photo-oxidation under the present conditions also indicates three such positions; thus the unit molecular ratio of pressure decrease to pigment concentration observed normally for chlorophyll, has been shown to be increased to three on conversion to the magnesium-free pheophytin.[13c] While these ratios are capable of more than one interpretation, depending on whether the pressure decreases observed refer solely to an absorption of oxygen or to an oxidation involving the production of some resultant gas or gases, the present work in each case shows that the final product of the reaction is a hydroperoxide. The amount of peroxidic oxygen detected is equivalent to approximately 80% of the pressure decrease observed (pp. 81-87); this result was obtained for oxidations on glass only, some difficulty being observed in estimating hydroperoxide when thallous bromide was used. For chlorophyll then it may be concluded that, under normal conditions, illumination results in the absorption of oxygen with the formation of a peroxide, in which each molecule contains one molecule of oxygen in a hydroperoxide group.[13]

If, as has been suggested, formation of this hydroperoxide takes place at the C_{10} atom of the molecule, the properties of this position are such that they would be consistent with an oxidation which was reversible in nature. Thus enolisation occurs readily at this position, and on the assumption that such a change may be brought about by illumination, the sequence of reactions

represented in the previously proposed scheme (p. 4) has been interpreted chemically by the following mechanism -



where (a) is a possible configuration of the 'reversible oxide', and (b) the probable hydroperoxidic end-product, only the relevant portion of the chlorophyll molecule being shown here. The stability of the final compound would be explained by a resonance association of the hydrogen atom with either of the adjacent carbonyl groups.

Since the process of allomerisation of chlorophyll has been postulated,[21] though not proved, to involve an oxidation at the C_{10} atom of the molecule, it is of interest to note that experiments carried out on allomerised chlorophyll under the conditions of the present work, showed a photo-oxidation similar to that for normal chlorophyll, with unit molecular ratio and partial rate reversal after evacuation.[57]

With regard to the other possible oxidation centres of the chlorophyll molecule, recent investigation[57] has shown that under similar conditions the oxidation features of the separated chlorophyll a and b fractions are the same as those of the mixture, the molecular ratio on thallous bromide being unity and rate reversal in evidence after evacuation; from this it would appear that the aldehyde group of chlorophyll b is not concerned in the reaction. Further evidence indicates that the two 'extra' hydrogen atoms also are not involved, and points instead to the

phytol side chain as the initial position of photo-oxidation of chlorophyll under the above conditions. An oxidation in this position alone would not, however, be consistent with the marked decrease in the intensity of the red absorption band of chlorophyll which has been observed to accompany this reaction.

While it is unfortunate that in the last section of the present work - where the effect of added adsorbents on the general features of the photo-oxidation has been investigated - a certain disagreement with the earlier findings is apparent, it seems quite reasonable to assume that the differences observed are due to a progressive deterioration of the material under investigation. Thus whereas previous to this, results have been consistent, showing a unit molecular ratio of pressure decrease to chlorophyll concentration, independent of the solid used to form the film, in this particular section the ratios observed are all greater than unity and vary to some extent with the substrate. As mentioned previously, such an increase in ratio is consistent with the presence in the chlorophyll of an appreciable amount of pheophytin, the molecular ratio found with this degradation product alone being equal to three. In general, however, the results of this work show that for all the preparations examined the value of the molecular ratio is greatly increased in the presence of soda lime and/or phosphoric oxide; some factor other than the simple uptake of oxygen would thus appear to be involved in their photo-oxidation. Further work is obviously required on this aspect of the subject using chlorophyll preparations which are known to be intact, but several interesting points arise from a consideration of the results obtained here, and these may now be discussed.

Two possibilities must be taken into account in considering the effect of suitable adsorbents on a reaction involving the uptake of the sole reacting gas with the production of another resultant gas or gases - thus apart from the alteration in the overall pressure change which must result, the course taken by the reaction may be unchanged and the final solid product the same as that formed in the absence of the adsorbent; on the other hand it is possible that the gaseous products have a retarding or inhibiting effect, and that by their removal the reaction is allowed to proceed further.

In the present instance the observed effects of soda lime and phosphoric oxide indicate that carbon dioxide and water are products of the photo-oxidation of the pigments examined. For chlorophyll the maximum molecular ratio observed (5·89) corresponds to the absorption of almost six molecules of oxygen per molecule of pigment, this result being obtained with spinach chlorophyll in the presence of a mixture of phosphoric oxide and soda lime. With phosphoric oxide alone as adsorbent the value observed was almost five, while with soda lime by itself results varied from 4·27 to 5·79, the highest ratio being observed after evacuation. If, as already suggested (p. 92), evacuation corresponds to a more efficient adsorber - possibly of water vapour - then it appears that approximately one molecule of gas adsorbable only by the soda lime is produced by the oxidation. In view of the evidence of degradation to pheophytin observed with the chlorophyll preparations examined, it is of interest to note that oxidation of this compound itself in the presence of soda lime gave a molecular ratio of about 5·5.[57] It is difficult to see how the gases indicated by these experiments, and water in particular,

can be present in normal oxidations since the molecular ratios observed are more or less exact integers, and have been shown to be not appreciably affected by their addition to the system. In general, however, it would appear from these results that an oxidation considerably more complex than that suggested by the simple reversible oxide theory is here involved.

The amount of peroxidic oxygen detected in the oxidised pigment after illumination in the presence of adsorbents, was equivalent to only a small percentage (approximately 15) of the pressure decrease observed, a result in contrast to the almost complete equivalence (approximately 80%) found after normal oxidations. Comparison of the actual percentages obtained, however, with the corresponding molecular ratios for the experiments in question, shows that, within experimental error, the results are consistent with the presence in each molecule of the product of approximately one molecule of hydroperoxidic oxygen. Thus, in general for a molecular ratio of six, the percentage of the pressure decrease which would correspond to one molecule of hydroperoxide per molecule of the product, in consideration of the approximately 80% equivalence observed with the unit ratio, would be some $\frac{80}{6} \div 13\%$. The final product appears, therefore, in this respect to be the same as that formed under normal conditions. Since the ratios observed in the absence of adsorbents with the chlorophyll samples in question indicate an appreciable degradation to pheophytin, and it has been shown (p. 87) that with this compound, which gives a molecular ratio of three, the amount of peroxidic oxygen detected is again equivalent almost completely to the pressure decrease observed,

in the experiments with adsorbents a higher percentage of peroxidic oxygen might have been expected. The results actually obtained must indicate then, that whereas the normal 'chlorophyll' hydroperoxide is formed and remains intact during the prolonged illumination required in the oxidations with adsorbents present, those of the imino positions - if formed under such conditions - must undergo some subsequent change whereby they are decomposed.

With carotene the results obtained under conditions similar to those used with chlorophyll seem to show that in this case also the oxidation is complex. In view of this, and of what has already been said about chlorophyll, it appears unlikely that the theory of the simple reversible nature of these pigment oxidations can still be supported; a more general consideration of the results of the present investigation may, therefore, now be undertaken.

The view that the unit molecular ratio and the increased rate after evacuation observed in photo-oxidations under the present conditions, could be ascribed to the formation of a reversible peroxide, was put forward originally on the grounds that (a) it seemed unlikely that dissimilar molecules such as chlorophyll and carotene, should have the same oxidation characteristics if a complex oxidation is taking place, (b) a likely product of complex oxidation in both cases is water, where the production of water in an amount of the required magnitude would be attended by such adsorption by vessel walls and by the solid preparation that unit ratios would not be found (these points having been confirmed experimentally by the direct rapid introduction of water vapour, and by the independent investigation of the photodecomposition of oxalic acid on thallous bromide,

where water is actually formed), and (c) this conception of reversibility was not in disagreement with Kautsky's[17] claim that the kinetics of chlorophyll fluorescence indicate the initial production of an unstable oxygen complex, followed by a non-dissociable peroxide on further illumination, as well as with Baur's[18] finding that carotene peroxide is formed only in light with a partially reversible formation.

While the initial results of the present work seemed to support these views, as already discussed, it is evident now that several other factors are involved and that this theory can no longer be upheld. Neither the evolution of oxygen nor the regeneration of chlorophyll have been proved, though in both cases reasons have been given (pp. 75,84) as to why such features have not been observed. It is true that below a certain pressure of oxygen the rate of oxidation for chlorophyll reaches a very low value explainable by the reversible oxide theory, but experimentally there is only the fact that for such conditions one relatively rapid rate is replaced at lower pressures by a relatively low one. Any sharp change between such rates is difficult to observe experimentally. At the same time carotene (p. 35) shows no similar sharp 'limit', although the increase in rate after evacuation with this compound is appreciable; a rapid conversion of 'reversible' to 'stable' oxide would here mean little increase in rate on evacuation. Even if the idea of a reversible oxide be given up as an explanation of the increased rates observed on evacuation, it seems unlikely that a similar explanation could be retained for the very first stage of the oxidation because no limit is found for chlorophyll in the

presence of phosphoric oxide (p. 62). It seems more likely in fact that, in spite of the overall resemblance between the results for chlorophyll and carotene, both are more complex than is suggested by the simple reversible oxide theory. Similar remarks apply in the case of pheophytin.

An essential feature of the present results is the difference in rates of pressure decrease for apparently similar total pressures, both for chlorophyll and for pheophytin, when such pressures are relatively low. This can only be explained by the presence of some retarding reaction which, from the low temperature coefficient (pp. 57,79) is probably photochemical. Such a retarding product is not normally non-volatile (pp. 109-111)[46]; indeed, Lonie showed that a reduction in oxygen pressure from 100 to 50 mm. gave, on subsequently restoring the 100 mm. pressure, an appreciably increased rate. Any such product is most likely to be colourless, or at least the absorption of visible light at such low pressures will be small; a reaction sensitised by thallous bromide would then be expected. The relation between primary and secondary reactions will not be a priori the same on thallous bromide as on glass. In view of this, pressure limits observed on thallous bromide, thallous iodide, and glass, would be expected to be different, and this is indeed the case for most of the preparations examined.

In devising a scheme of oxidation reactions for chlorophyll which more nearly meets the present requirements, the molecular ratios observed under the various conditions and the corresponding amounts of hydroperoxide formed are to be noted. Thus in the absence of adsorbents, where unit molecular ratio is observed,

the amount of hydroperoxide detected is some 80% of the pressure decrease, if that decrease is considered as oxygen. While results in the presence of adsorbents are unfortunately complicated by the degradation of the pigment which had apparently set in on standing, and the light increased pheophytinisation on glass (p. 97), the most reliable value for the molecular ratio seems to be that of spinach chlorophyll in the presence of soda lime and phosphoric oxide, equal to 5·89: under such conditions the amount of hydroperoxide expressed as a percentage of the total pressure decrease is 15%, in agreement again with the assumption of one molecule of oxygen present as hydroperoxide. The amount of non-volatile hydroperoxide is thus independent of the presence of adsorbents, which must be taken to act on the volatile ratarding product. A possible general scheme is then as follows -

$$\text{Chl} + 3O_2 \longrightarrow \text{Chl.}'O_2 + A \qquad (1)$$

$$A + nO_2 \longrightarrow B + (n+2)P \qquad (2)$$

where Chl.$'O_2$ represents the solid hydroperoxide, A is a volatile gas product, B is a possible non-volatile product, not peroxidic, and $(n+2)P$ represents $(n+2)$ gas molecules of some kind.

The total pressure decrease is then $(3+n)-(n+2) = 1$ molecule per molecule of chlorophyll, the hydroperoxide is equivalent in oxygen used to this pressure decrease, and the second reaction represents a retarding reaction, $(n+1) \longrightarrow (n+2)$, removal of A causing an increased rate of pressure decrease. The factor 3 of equation (1) is then a minimum value.

There is no change in pressure in the dark; all such reactions are therefore conditioned by light exposure. If it is

assumed that the rates of reactions (1) and (2) depend on I_{abs} for each species, and it is taken that I_{abs} is proportional to the concentration, then the system can be represented by the consecutive series of unimolecular reactions

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

which for the above scheme may be written

$$A \xrightarrow[k_1]{3O_2} B \xrightarrow[k_2]{nO_2} C + (n+2)P$$

where k_1 and k_2 are the two velocity constants.

Let the initial concentration of chlorophyll (A) at time $t = 0$ be a. At any time t we have the concentrations C_A, C_B and C_C for A, B and C respectively, and since the reactions are consecutive

$$a = C_A + C_B + C_C$$

Now

$$-\frac{dC_A}{dt} = k_1 C_A \quad \therefore \quad C_A = ae^{-k_1 t}$$

and

$$\frac{dC_C}{dt} = k_2 C_B$$

$$\therefore \quad \frac{dC_B}{dt} = -\frac{dC_A}{dt} - \frac{dC_C}{dt} = k_1 C_A - k_2 C_B$$

$$\therefore \quad C_B = \frac{ak_1}{(k_2 - k_1)}\left(e^{-k_1 t} - e^{-k_2 t}\right)$$

$$\therefore \quad C_C = a - (C_A + C_B)$$

$$= a - ae^{-k_1 t} - \frac{ak_1}{(k_2 - k_1)}\left(e^{-k_1 t} - e^{-k_2 t}\right)$$

But

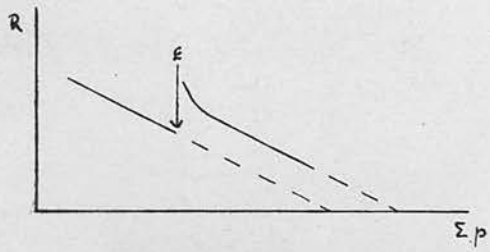
$$\frac{dp}{dt} = -3k_1C_A + (n+2)k_2C_B \quad \text{where } p = \text{total pressure}$$

$$= \frac{(n+2)ak_1k_2}{(k_2 - k_1)}\left(e^{-k_1t} - e^{-k_2t}\right) - 3k_1ae^{-k_1t}$$

$$= \frac{ak_1}{(k_2 - k_1)}\left[(3k_1 + (n-1)k_2)e^{-k_1t} - (n+2)k_2e^{-k_2t}\right]$$

$\frac{dp}{dt}$ is the rate of pressure decrease and is, therefore, determined only by a, the initial concentration of chlorophyll, and by constants. The rate curve will therefore be the same shape and slope after evacuation as before, since C_B is not involved.

Further, it follows that immediately after evacuation, in fact before B accumulates, there should be a rapid fall in rate, the linear decrease being attained when equilibrium conditions are reached: this will not be evident in the first illumination since reaction (1) stages must have time to develop. The form of the curves before and after evacuation should then be



The proposed mechanism conforms then to the experimentally observed features of the photo-oxidation.

Determination of the detailed nature of such a scheme, and of the characters of the gas molecu les produced, must await further investigation, but in conclusion it may be claimed that the present work has considerably extended and classified the conceptions and assumptions of the previous workers in this somewhat complicated field. The effects of the use of thallous

bromide as substrate in place of glass have been more clearly related to the corresponding features of the rate curves; the importance of the use of freshly prepared specimens and their possible alterations during illumination, apart from oxidation, have been emphasised; and in particular it is believed that the evidence here reviewed shows that the simple reversible oxide theory cannot be satisfactorily supported.

SUMMARY

Examination has been made of systems of chlorophyll and related compounds, as deposited on various solids, by direct measurement of the pressure changes involved on illumination by visible light in oxygen.

Chlorophyll and carotene show a pressure decrease under such conditions which corresponds, by a rate extrapolation method, to a molecular ratio of pressure decrease to pigment concentration of unity; after evacuation, increased rates are in evidence. The view that such behaviour can be ascribed to a simple oxidation which is reversible in nature, appeared to be borne out by the results obtained at low oxygen pressures ($< 0{\cdot}5$ mm.); thus for chlorophyll it was shown that there exists a pressure limit below which in general the rate of oxidation becomes very slow. Similar behaviour was observed with pheophytin. In the presence of phosphoric oxide, however, no marked pressure limit was found, while in this pressure region a variation in rate for apparently similar total pressures was evident, which could only be explained by the presence of some retarding reaction.

At high oxygen pressures (100 mm.) extrapolated pressure decrease values were observed in the presence of soda lime and/or phosphoric oxide, which corresponded to molecular ratios greater than unity, the maximum ratio obtained for chlorophyll being approximately six. Similarly increased ratios were found with carotene.

The general features of the oxidations were shown to be the same when the pigments were adsorbed on glass as on thallous

bromide, rates at high oxygen pressures then being slower; the thallous bromide is regarded as a photosensitiser.

A colorimetric method of estimating the amount of peroxidic oxygen present after the illumination of such pigment systems in oxygen was developed. For chlorophyll the amount of hydroperoxide found after oxidations in the absence of adsorbents was some 80% of the pressure decrease, if that decrease be considered as oxygen, while in the presence of adsorbents approximately 15% was found; these results are consistent with the assumption of one molecule of oxygen present in the product as hydroperoxide in each case.

In consideration of the evidence presented, it is believed that the simple reversible oxide theory cannot be satisfactorily supported. A possible general mechanism involving consecutive reactions has been proposed to account for the main features of the photo-oxidation.

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